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MEASUREMENT OF MAMMALIAN CELL ADHESION

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Dedicated to my parents and my wife Rubina Sarwar

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ABSTRACT

Cell adhesion is an intricate process, involving proteins of the extracellular matrix, cell surface receptors for these proteins and a complex interplay of physical, biochemical and cytoskeletal events. This may affect the cell itself, the substrate to which the cell will attach or the environment in which cells grow. Although morphological studies of cell adhesion have been made in exquisite detail, such results are only qualitatively useful in predicting cell-substratum compatibility. Usually a quantitative cell adhesion is measured by subjecting the attached cells to the hydrodynamic forces for detachment from the surface Unfortunately a very few quantitative methods of cell adhesion measurement have been proposed but they all suffer from the need for complex equipment Moreover, the reproducibility of these methods remained a dream due to uncontrolled, limited (in their ranges) and indefinable forces produced by the devices used in these methods. In the present work these dreams became reality when a simple and reproducible cell adhesion measuring device "Microflow chamber" has been developed which produces a wide range of hydrodynamic forces for cell detachment.

A convergent channel is a unique feature of the "Microflow chamber" which distinguishes this device from previously developed methods. That is in the Microflow chamber the physical conditions of the detachment assay are completely defined. It is pleasing about this device that it is able to take account of the adhesive potentials of the cell, substratum and the environment of the attached cells. The reproducibility of this device was extraordinary as it was amenable to make difference among the adhesion strength of different cell lines, on different substrates and in different environments. That is after taking from liquid nitrogen within 72 hours every cell line tested has its specific and constant adhesion strength (in terms of critical shear) value. The majority of the cell lines tested in this work show their specific and constant shear value even after 6 months of taking from liquid nitrogen.

The substrates on which cells grow also exert some effect on their adhesion strength. In particular fibronectin coated substrata were found to possess a catalytic

activity for enhancing the adhesion strength of L929 cells. As cell adhesion is an active phenomenon the adhesion strength was also found dependent on the endogenous production of adhesion proteins without which cells remained confined into the first phase and never entered into the second phase of cell adhesion (two phases are proposed in the present study). In fact it is due to the Microflow chamber that in the present work a considerable understanding of the cell adhesion strengthening phenomenon has been reached which can be considered a start of the complete understanding of this complex phenomenon.

ABBREVIATIONS

-ODhbt	dihydro-oxobenzotriazine
-OPfp	pentafluorophenyl derivatives
^{125}I	iodine 125
^{14}C	carbon 14
^{35}S	sulphur 35
BHK	baby hamster kidney
BSA	bovine serum albumin
c.s.s.	critical shear stress
CAM	cell adhesion molecule
cAMP	cyclic adenosine monophosphate
CELL-CAM	cell-cell adhesion molecule
CHL	chinese hamster lung cells
CPM	counts per minute
DMEM	Dulbecco's modification of Eagle's medium
DMF	dimethylformamide
ELAM	endothelial leukocyte adhesion molecule
EMA	ethyl methacrylate
FCS	foetal calf serum
Fmoc	9, fluorenylmethyloxycarbonyl
Hela	cervical carcinoma cells of Helen Lane
HEMA	hydroxymethyl methacrylate
HEPES	(N-2-hydroxyethyl piperazine- N-2-ethanesulphonic acid
HOBT	hydroxybenzotriazole
HPLC	high performance liquid chromatography
HS	horse serum
L-CAM	liver cell adhesion molecule
L929 CELLS	mouse L strain (clone 929)

LDV	-leucine-aspartic acid-valine
MDCK	Madine Darby Canine kidney cells
MEM	minimal essential Eagle's medium
MMA	methyl methacrylate
Mtr	methoxytrimethylbenzenesulphonyl
N-CAM	neural cell adhesion molecule
Ng-CAM	neuron glial cell adhesion molecule
NM	nicotinamide
Nm^{-2}	Newton per metre square
PBS	phosphate buffer saline
REDV	-arginine-glutamic acid-aspartic acid-valine-
RGDS	-arginine-glycine-aspartic acid-serine-
RPMI 1640	Roswell Park Memorial Institute modification 1640
RYVVLPR	arginine-tyrosine-valine- valine- leucine-proline-arginine
t-AMYL	tertiary amyl
TFA	trifluroacetic acid
WRC	Walker rat carcinoma cells
YIGSR	tyrosine-isoleucine-glycine-serine-arginine

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CHAPTER 1 INTRODUCTION

1. CELL ADHESION

In reference to cell in cell biology "adhesion" has been a portmanteau word applied to the interaction of cells with each other and with surfaces. A variety of these definitions include early and late cell binding events and the specific and non specific adhesions. Cell adhesion phenomena can be seen in a wide range of taxa, from prokaryotes to eukaryotes and from unicellular organisms to metazoans. The study of the differential binding and embryonic development of cells in multicellular organisms began in 1907 when Wilson(1907) showed that mechanically dissociated and mixed cells of two different species of marine sponges sorted themselves out to produce two types of organisms, each consisting of the cells of only one species. A similar approach was later applied by Holtfreter (1948) who showed that dissociated embryonic cells derived from different tissues could sort themselves out from regions and structures resembling parent tissues. These experiments showed that there is a selective adherence among cells of various types.

This paradigm was extensively employed by Moscona et al (1962) to show similar phenomena from chicken and mice. Various attempts were subsequently made to develop more direct assays of cell- cell adhesion to isolate molecular fractions that might be responsible for different selectivity or specificity (Frazier and Glaser, 1979; Hausman and Moscona, 1976). More recently, these issues have been widely addressed and it is only within the last decade that details of the molecular basis of cell adhesion are beginning to emerge (Edelman 1985a, Edelman, 1985b; Edelman, 1988, Nagafuchi & Takeichi, 1988, Nelson & Hammerton, 1989).

According to publications to date cell adhesion can be considered as an intricate process, involving proteins of the extracellular matrix, cell surface receptors for these proteins and a complex interplay of physical, biochemical and cytoskeletal events (Buck & Horwitz 1987; Vasilev, 1985; Morrow et al, 1989). Two approaches to studying the problem of cell adhesion have been developed:

(1) The adhesion of cells to each other

(2) The adhesion of cells to extracellular substrates

These will now be discussed below.

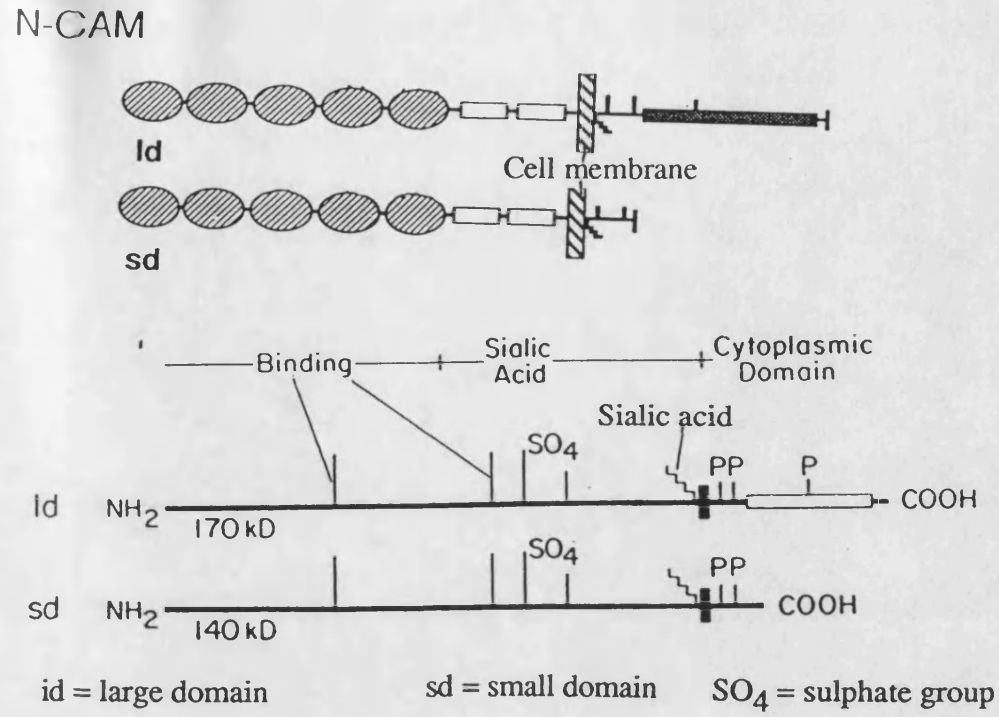
1.1. CELL--CELL ADHESION.

In cell to cell adhesion, at the first encounter between one cell and another there is an initial binding event. This initial binding is brought about by a non covalent molecular interaction between component(s) of the extracellular matrix and receptors on the cell surface.

Many cell surface molecules have been implicated in cell adhesion, presumably such molecules are involved in initial binding. These have been referred to as cell adhesion molecules(CAMs)(Edelman et al, 1987, Friedlander et al, 1989). CAMs were first identified by means of immunological based assays in which specific antibodies, capable of blocking cell adhesion, were used to purify cell surface molecules as putative CAMs. A number of these molecules have been detected. Among them the most studied are neural cell adhesion molecule(N-CAM), neuron glial cell adhesion molecule(Ng-CAM) and liver cell adhesion molecule(L-CAM). All the molecules that have been characterized are shown to be large glycoproteins.

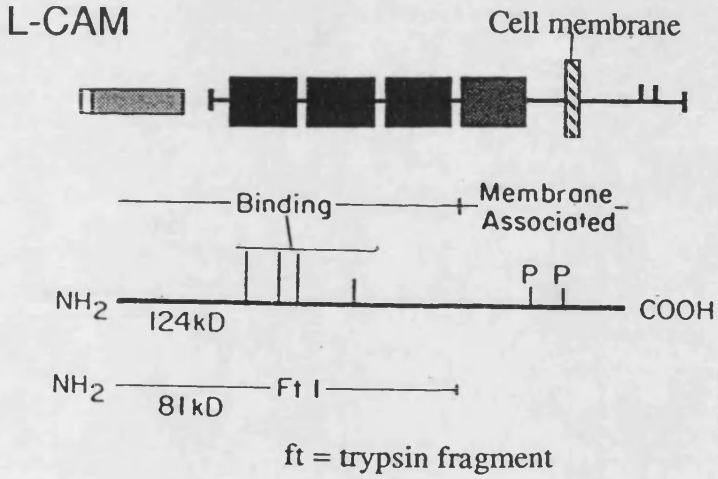
1.1.1. N-CAMs

NCAMs consist of one or more structurally related polypeptide chains (figure 1.1). These appear in various forms with regard to glycosylation i.e highly sialylated and less sialylated forms of N-CAMs exit. The highly sialylated form, which dominates during the embryonic period contains polysialic acid. The less sialylated form dominates in adult animals and has no polysialic acid. Moreover the binding rate of the less sialylated form is much higher than that of the highly sialylated form. It is suggested that the N-CAM on one cell binds to an N-CAM on adjacent cell (Rutishauser, et al, 1982; Cunningham et al, 1987; Santoni et al, 1989; Lanier et al, 1989).



id = large domain sd = small domain SO_4 = sulphate group

P = phosphate group



ft = trypsin fragment

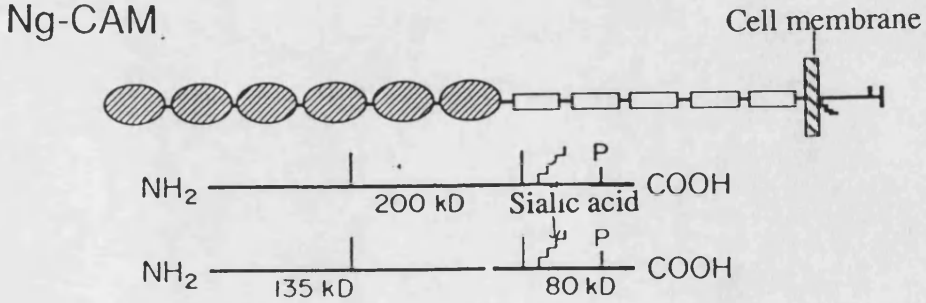


FIGURE 1.1.

STRUCTURAL FEATURES OF SOME OF THE PROMINENT CELL-CELL ADHESION MOLECULES. DETAILS MAY BE SEEN IN THE TEXT.

1.1.2. L-CAM

L-CAM was originally isolated from embryonic chick liver but has been found in all types of epithelia. It consists of one polypeptide chain that is amino glycosylated on the N-terminal end. The C-terminal portion of the polypeptide chain is associated with the membrane (figure 1.1). It is not known if the binding reaction is homophilic or heterophilic i.e. if the molecule binds to itself on the other cell or to a ligand of different identity (Thiery et al, 1984; Cunningham et al, 1984; Hatta et al, 1985; Obrink, 1986).

1.1.3. Ng-CAM

Ng-CAM is a secondary CAM, consists of a large glycoprotein (200Kd) that appears to be posttranslationally cleaved into two components of 135Kd and 80Kd (figure 1.1). It has been implicated in neuron-neuron and neuron-glial cell binding. It has an unidentified ligand to which it binds on opposed cell, therefore the binding must be heterophilic (Brachenbury et al, 1981; Grumet et al, 1984; Kruse et al, 1985). Many other adhesion related molecules have been found. These include, cadherin, cell-CAM and ELAM.

1.1.4. CADHERIN

The cadherins are a family of homologus cell surface glycoproteins which mediate Ca^{2+} dependent cell-cell adhesion. This family includes: E-cadherin, N-cadherin, P-cadherin. They are found on epithelial cells, nerve cells and on the cells of the placenta respectively (Shirayoshi et al, 1983; Yoshida et al, 1984; Hatta et al, 1987; Takeichi, 1988; Nose et al, 1988).

1-1.5. CELL-CAM

Cell- CAM is involved in cell-cell adhesion and compaction of early embryos. It is present in early embryo and several epithelia(Damsky et al, 1983; Hixson et al, 1985; Vestweber et al, 1985)

1.1.6. ELAM (endothelial leukocyte adhesion molecule)

ELAM is a member of integral membrane adhesion proteins which share a common extracellular domain orgnisation consisting of an NH_2-terminal lectin like segment. It serves as an inducible receptor for Neutrophil binding to endothelial cells via sialic acid groups (Walz et al, 1990).

Apart from these molecules an important event that can strengthen an adhesion is the formation of specialised cell junctions. Cell junctions can be classified into three functional groups:

1. Tight junctions seal cells together in an epithelial cell sheath in a way that inhibits even small molecules from leaking from one side to the other (Simons & Fuller, 1985; Madra, 1988).

2. Anchoring junctions consist of adherens and desmosomes. Adherin junctions are button like points of intracellular contact that rivet cells together in a variety of tissues, most of which are epithelials. They also connect actin filaments to the extracellular matrix. Desmosomes are connection sites for intermediate filaments (Garrod, 1986).

3. Gap junctions play an important role in cell coupling in embryogenesis. For instance the cells within each group remain coupled via gap junctions with each other and so tend to behave as a cooperative assembly (Pitts & Finbow, 1986; Loewenstein, 1987)

Thus cells in tissues are linked to each other and to the extracellular matrix via these molecules.

The present work is primarily concerned with the second approach of cell adhesion study i.e. cell to substrate adhesion.

1.2. CELL-SUBSTRATE ADHESION

This is the subject of the present study. It is conveniently divided into following identifiable stages:

1 Protein adsorption on the surfaces

2 Cell attachment

3 Spreading

4 Growth

Each of these steps , perhaps with the exception of number 1, may require, like cell-cell adhesion, an array of factors from cell bound proteins to proteins in the solution to metal ions. Cellular metabolism also plays a central role in 2,3 and 4.

1.2.1. PROTEIN ADSORPTION ON THE SURFACE

The first reaction that occurs when a virgin surface (plastic/metal/glass) is immersed in a solution containing proteins is that the latter irreversibly bind and denature onto the surface. (Soderquist & Walton, 1980; Castillo et al, 1984; William et al, 1986; Absolom et al, 1987). The protein adsorption studies date back at least to 1925 and thereon almost all published research work has invoked a Langmuir type adsorption process (Hitchock, 1925; Kemp & Rideal. 1934; Absolom et al, 1987) Thus the act of pouring medium containing serum into a culture bottle causes a rapid deposition of a protein layer on the flask. This layer may only be fractions of a micrometer in thickness (Castillo et al, 1986; Anderson et al, 1990).

Nearly all mammalian cell adhesion to synthetic substratum is aided by adsorbed proteins and is actively receptor mediated (Evans, 1985; Vogler, 1989; Cozens-Roberts et al, 1990). After protein adsorption, if there are receptors for some of these adsorbed protein(s) on the cell surface and if the conformation of the adsorbed proteins is not so extensively altered by adsorption as to destroy the high ligand-receptor affinity, then cell adhesion can result (Schakenraad et al, 1987).

As stated earlier, adsorption of proteins to the surface is largely irreversible and much more rapid than contact of the cell to the surface so that cells interact with an interface of previously adsorbed proteins rather than the original form of substrate(Baier & Dutton 1969; Horbett & Weathersby, 1981; McAuslan et al, 1988; Lee et al, 1991). Recently Schakenraad et al(1989) suggested that protein adsorption

is completed within minutes and must precede attachment and spreading which generally require several hours.

Under static conditions , transport of proteins toward a substratum is controlled either by sedimentation, diffusion or convection. The properties of the substrate surface e.g. hydrophilicity/hydrophobicity and surface charge are assumed to regulate the amount and surface structure(conformation) of adsorbed proteins.(Uyen et al 1990; Fabrizius-Homan & Cooper, 1991).

Wachem et al(1985) showed that a moderate wetability of a surface is a precondition for maximal cell adhesion in the presence of serum in the culture medium. Wetability was defined by the measurement of contact angle of the material. For example cellophane was found to be the most hydrophilic polymer with a contact angle of 16° and flouroethylenepropylene polymer was the most hydrophobic polymer with a contact angle of 102°. Tissues culture plastic with a contact angle of 35° was considered a moderately wettable polymer. Moderately wettable surfaces showed a good cell adhesion whereas both more hydrophilic and more hydrophobic polymers showed poor cell adhesion.(Wachem et al, 1987).

The mechanism by which the surfaces control the amount and conformation of the proteins is still unknown.

Apart from wetability, surface charge is reported to play a part in controlling the protein adsorption on the surfaces (Maroudas, 1975; Sugimoto, 1981). For instance, protein adsorption onto the negatively charged poly HEMA (hydroxyethylmethacrilate) is supposed to be slight and reversible and no cell adhesion was found to occur onto this surface(Wachem et al, 1987; Gerson & Scheert, 1988; Schakenraad & Busscher, 1989). However, maximal adhesion of fibroblasts upon positively charged poly-HEMA was observed by Hattori et al(1985).

Despite these reports a considerable confusion exits in the literature concerning protein adsorption and cell adhesion. For example fibronectin (a cell adhesion protein) adsorption has been the subject of contrary reports. Klebe et al (1981) demonstrated that the substrates capable of binding fibronectin shared one

common surface property; namely, they were all hydrophobic and they all bound protein non specifically. On the other hand those substrates that did not bind fibronectin were all hydrophilic in nature.

In findings contrary to the above, Grinnel & Feld (1981) suggested that the fibronectin adsorbed on a hydrophilic substrate has a different orientation than the fibronectin on a more hydrophobic substrate. This author also suggested that fibronectin adsorbed on the hydrophilic substrate was biologically more active. Thus one must compromise on that, the moderate wetability, the better the cell adhesion (Lydon et al (1985). The surfaces which are not moderately wettable (defined earlier) and do not have high surface charge must be treated chemically or physically i.e. treatment with oxidising agents or electrical glow discharge to render them suitable for cell adhesion.

Martin and Rubin(1974) treated polystyrene with sulphuric acid and exposed it to u.v. light. This resulted in a suitable surface for the adhesion of fibroblast cells. In fact this treatment was thought to lead to the sulphonation of the polystyrene with a consequent increase in the number of charged groups per unit area which in turn supported cell adhesion. Later Curtis et al (1983) discarded the assumption that sulphate groups accounted for enhanced cell adhesion to polystyrene in favour of the idea that sulphuric acid treatment caused hydroxylation of aromatic groups on the surfaces. These hydroxyl groups, emerged on the surface, were suggested to be involved in cell adhesion. Three years later, Curtis et al, 1986; and Smentana et al, 1990) suggested that an increase in the adhesion densities of hydrooxyl groups was entirely responsible for cell adhesion whereas fibronectin(a prototype adhesion protein) is only an activator of cell adhesion.

Lydon et al (1985) responded to the claims of Curtis et al (1983) and put a question mark on the hydroxyl groups in cell adhesion Lydon et al raised the point that if the hydroxyl groups are responsible for cell adhesion why does poly- HEMA with its higher density of hydroxyl group, not support cell adhesion and poly methyl methacrylate (MMA) which has no hydroxyl group supports cell adhesion. This issue

is still a matter of debate among cell biologists. According to Revel and Wolken (1973) cellular protein synthesis as well as protein(s) released from dying cells during incubation in a protein free media could form an extracellular matrix (ECM) even in the absence of exogeneously added protein(s). It can be concluded that proteins are present in almost every situation where the substrate comes into contact with the cells. Modifications of commercial cell attachment substrates by electrical glow discharge, simply changes the pattern of protein adsorption (Blais et al, 1974). In no described case does the cell actually touch the surface and attach except through these intermediate adsorbed protein(s). With regard to the latter, a most significant development of the last decade has been the identification of a number of glycoproteins and collagens which were capable of adsorption on the surface and promotion of the cell adhesion. The first major glycoprotein of this type to be identified was fibronectin (Vehri & Mosher, 1978; Yamada et al, 1976,Yamada, 1983; Yamada & Olden, 1978) while later laminin and vitronectin have joined the list (Wewer, et al, 1987; Chi & Hui, 1989). More recently some new proteins such as thrombospondin (Santoro & Frazier, 1987), entactin (Chakravrti et al ,1990) and epilegrin (Carter et al, 1991) have also been discovered.

The most obvious properties of these molecules is to promote cell adhesion For the present discussion only fibronectin, vitronectin, laminin and the collagens will be described briefly.

1.2.1.1. FIBRONECTIN

Among all the adhesion proteins, fibronectin is structurally and functionally well understood. It is a large glycoprotein (440Kd) present in blood plasma and in tissues. Both plasma and cellular fibronectins are similar in structure and function; however, they are distinguished by certain physical properties such as solubility and mobility on SDS polyacrylamide gels. Under reducing conditions, plasma fibronectin migrates as a closely spaced doublet whereas cellular fibronectin migrates as a single band (Dufour et al, 1986). Three types of posttranslational modifications could in part

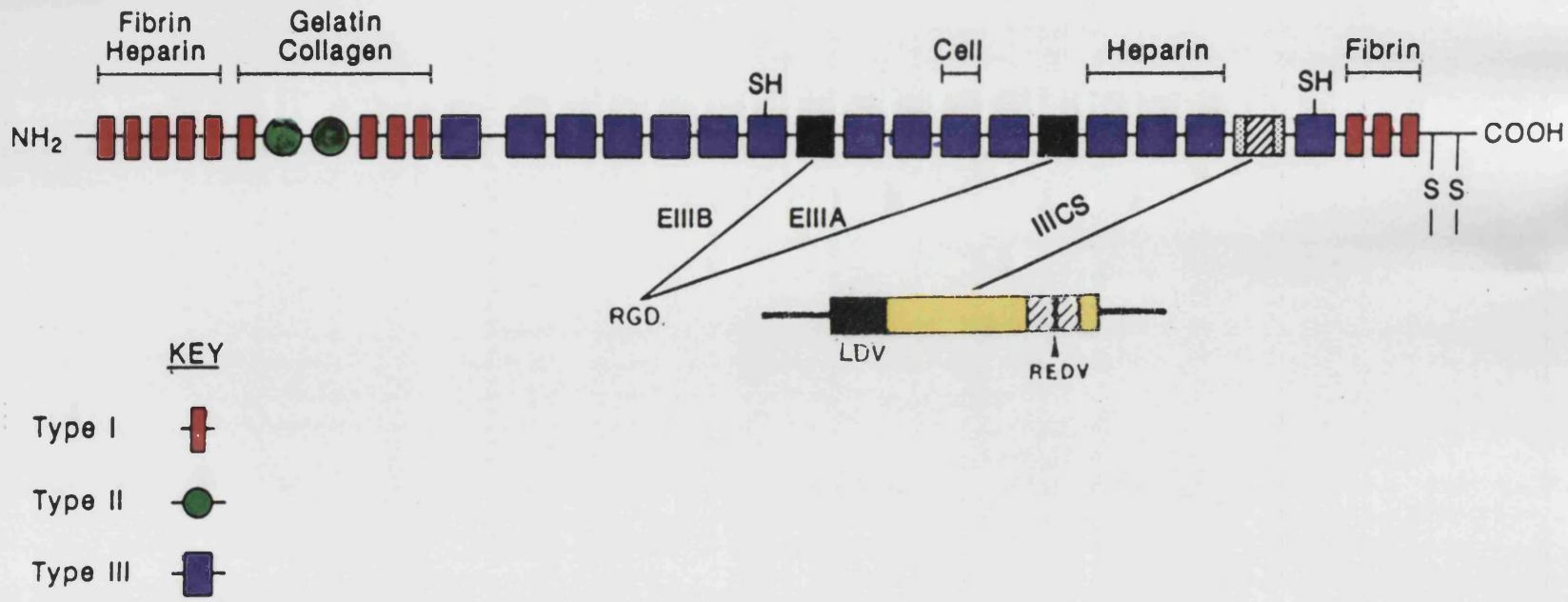


FIGURE 1.2.

SCHEMATIC REPRESENTATION OF THE DOMAIN STRUCTURE OF FIBRONECTIN.

THE KEY SHOWS DOMAIN ARRANGEMENTS IN THE FIBRONECTIN MOLECULE.

THE DIFFERENT CELL BINDING SITES ARE ALSO SHOWN. THE DETAILS MAY BE SEEN IN THE TEXT.

explain these differences; the variation in glycosylation, the degree of phosphorylation, and the presence or absence of sulphates. However the experiments aimed at elucidating the causes of differences in electrophoresis mobility have indicated that these post translational modifications can not solely be responsible for the differences between the plasma fibronectin and cellular fibronectin. For example it was suggested that differences between plasma fibronectin and cellular fibronectin reside in the size of the fragments, particularly the heparin binding domain (figure 1.2) (Paul & Hynes, 1984; Sekiguchi et al, 1985).

The protein contains two identical subunits, each of 240-250Kd, each linked together near their carboxyl terminal by two disulfides (figure 1.2) (Ehrismann et al, 1982; Ruoslahti et al 1986; Zardi et al, 1987). Fibronectin possesses many binding domains, each of which binds specifically to macromolecules such as heparin, proteoglycan, collagen, fibrinogen, actin and DNA. (Skorstengaard et al, 1986; Akiyama & Yamada. 1987; Wolf & Lai, 1990) (figure 1.2). In addition, fibronectin binds to the cell membrane via a cell binding site. It has been shown that at least three distinct types of internal amino acid sequence homology known as type (i), type (ii) and type (iii) exit along the molecule(Hynes, 1985; Gutman & Kornblit, 1987; Narasimhan & Lai, 1989).

The structure of the fibronectin molecule varies depending on the cellular source of the protein. These variations are due to alternative splicing of the fibronectin mRNA. Three regions of alternative splicing have been identified so far , two of them consist of splicing in or out of one type (iii) homology segment (iiiA or iiiB) whereas the third one has been termed connecting segment (iiics) (Zardi et al, 1987). Alternative splicing may effect the functions of fibronectin. Indeed the principal region of the human plasma fibronectin molecule mediating the adhesion of melanoma cells is the alternatively spliced type (iii) connecting segment (iiics) (Ruoslahti, 1988, and Oyama et al, 1989).

Formerly, the site in fibronectin that promotes cell attachment was found to be in the middle portion of the molecule, that is in one of what is now known to be the

type (iii) homology segment. This attachment site consists of a sequence of a 4 amino acids i.e.- arg-gly-asp-ser(RGDS) (fig. 1.2). The role of the RGD sequence as the recognition site was demonstrated by making progressively smaller fragments of fibronectin and by assaying for the cell attachment promoting activity It was found that RGDS containing peptides, in a soluble form could block adhesion of cells to fibronectin coated substratum, while coupled RGDS peptide to solid surfaces could promote cell adhesion to these surfaces (Pierschbacher and Ruoslahti 1984). The specificity of the RGDS sequence is extensively precise, substitution or deletion of a single amino acid abolishes the activity of this peptide (Hautanen et al,1989). For example, it was indicated that the serine residue of the tetrapeptide sequence (RGDS) can be cystine without any considerable loss of the activity of peptide but activity could be lost when Arg or Asp residues were selectively deleted or replaced by another amino acid. Even substitution of Asp acid with Aspargine ($AspNH_2$) greatly diminished the activity of tetrapeptide (Peirschbacher and Ruoslahti, 1984).

Humphries et al (1987) examined a series of overlapping synthetic peptides spanning the entire iiics region for their effects on F10 murine melanoma cell adhesion to the parent fibronectin molecule. Two non adjacent CS peptides CS1 and CS5 were found to inhibit melanoma cell adhesion to fibronectin. The CS1 peptide (Humphries et al, 1986) and CS5 peptides are located at the amino terminal ends of the alternatively spliced type (iii) connecting segment, respectively. Both contain the active sequence -arg-glu-asp-val- (REDV) (fig.1.2) . Recently Komoriya et al (1991) identified the minimal active (adhesive) amino acid sequence within the CS1 peptide. The deduced active amino acid sequence derived from carboxyl terminal of CS1 was -leu-asp-val- (LDV) (fig. 1.2).

Early experiments, correlating the extent of cell adhesion and cell spreading with adsorption of fibronectin to the culture substratum from growth medium containing serum suggested that fibronectin accounted for most of the cell adhesion activity in serum(Grinnell & Feld, 1982). In contrast to these studies a number of lines of evidence now suggest that under most common tissue culture conditions,

vitronectin is a more effective molecule than fibronectin for cell attachment and spreading.(Knox 1984). Structural and functional characteristics of vitronectin are given as below.

1.2.1.2. VITRONECTIN

For the first time Holmes (1967) showed that a serum fraction, isolated by making use of its affinity for glass beads, promoted the attachment and spreading of cultured cells. Barens & Sato (1980) subsequently showed that this serum fraction could be used as serum spreading factor to provide for cell attachment in serum free culture medium. The active component in this fraction was found to have an approximate M.wt>70,000. and that it was not fibronectin, or a fragment thereof. Latter this factor was identified as vitronectin (Hayman et al, 1983, 1985). Vitronectin promotes the attachment and spreading of a wide variety of both fibroblasts and epithelial cells. Vitronectin, like fibronectin, is a multifunctional protein with number of active sites (fig 1.3). These sites includes, a cell attachment site, a glycosaminoglycan binding site and a heparin binding site (Suzuki et al, 1985 and Izumi et al, 1988).

Human vitronectin is a monomeric glycoprotein of 75Kd. The amino acid sequence of this protein was deduced from cDNA and contains 459 residues (Jenne & Stanely, 1985). Heparin was stated to bind with an area of basic amino acids near the carboxyl terminal of vitronectin (Hayman et al, 1983). The cell binding site is located near the amino terminus. The minimum cell attachment site in this glycoprotein is similar to the cell attachment site in fibronectin i.e. RGDS (Ptyela et al, 1985a). Although both fibronectin and vitronectin support cell adhesion, Underwood and Bennet (1989) suggested that it is vitronectin that is the effective molecule for cell adhesion.

Two reasons for this findings were given, firstly, batches of foetal calf serum prepared for tissue culture are frequently clotted at $4^{o}c$ which leads to depletion of fibronectin. It was reported a range of 5-50 fold excess of vitronectin compared with

fibronectin amongst different lots of tissue culture grade foetal calf serum (Hayman et al, 1985). Secondly, the dependency of cells upon serum vitronectin, rather than fibronectin, in attachment, was shown to be due to less adsorption on the substratum in the presence of vitronectin.

The vitronectin was also able to coat the substratum efficiently in the presence of fibronectin and other proteins.(Underwood and Bennet, 1989). Stimulation of spreading by vitronectin has been postulated in a dose dependent manner. At a concentration of 18μg/ml more than 95% of BHK cells become completely spread on the substratum while at 10μg/ml only 15% of the cells were capable of spreading (Whately & Knox, 1980). In addition to fibronectin and vitronectin there is another type of glycoprotein which constitutes a major part of the ECM. This protein"laminin" appears to be most effective as an attachment protein for epithelial cells, however, it is also reported to support the attachment of some fibroblast cells (Codongo et al 1987).

1.2.1.3. LAMININ

Laminin, the major glycoprotein specific to basement membrane has multiple biological roles where it functions in cell adhesion, cell growth, morphology of the cells, differentiation and in matrix assembly (Kleinman et al, 1985 and Martin & Timple, 1987).

Laminin is a large(850Kd) cruciform shaped complex with three short and one long arm assembled from three different sub units i.e. A, B1 and B2 (fig. 1.4) (Engell et al, 1981, and Timple & Dziadek, 1986). The mechanism by which a cell adheres to laminin has not been completely elucidated. Two distinct proteolytic fragments of laminin P1 (pepsenic digestion product) and E8 (elastase digestion product) have been characterized for their activity in promoting the cell adhesion (Aumailley et al, 1987 and Goodman et al, 1987). These fragments respectively correspond to the central region and the end of the long arm. The domain in P1 responsible for attachment is

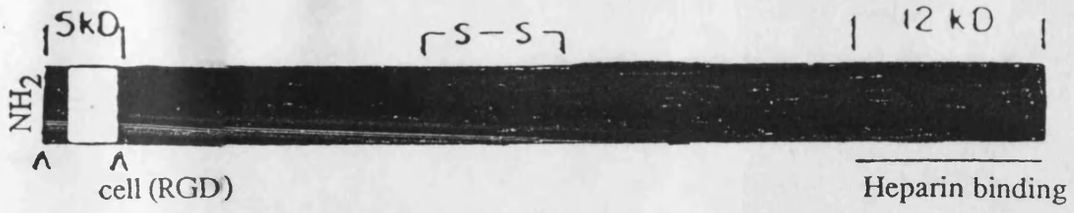


FIGURE 1.3.

DOMAIN STRUCTURE AND THE CELL ATTACHMENT SITE OF THE VITRONECTIN

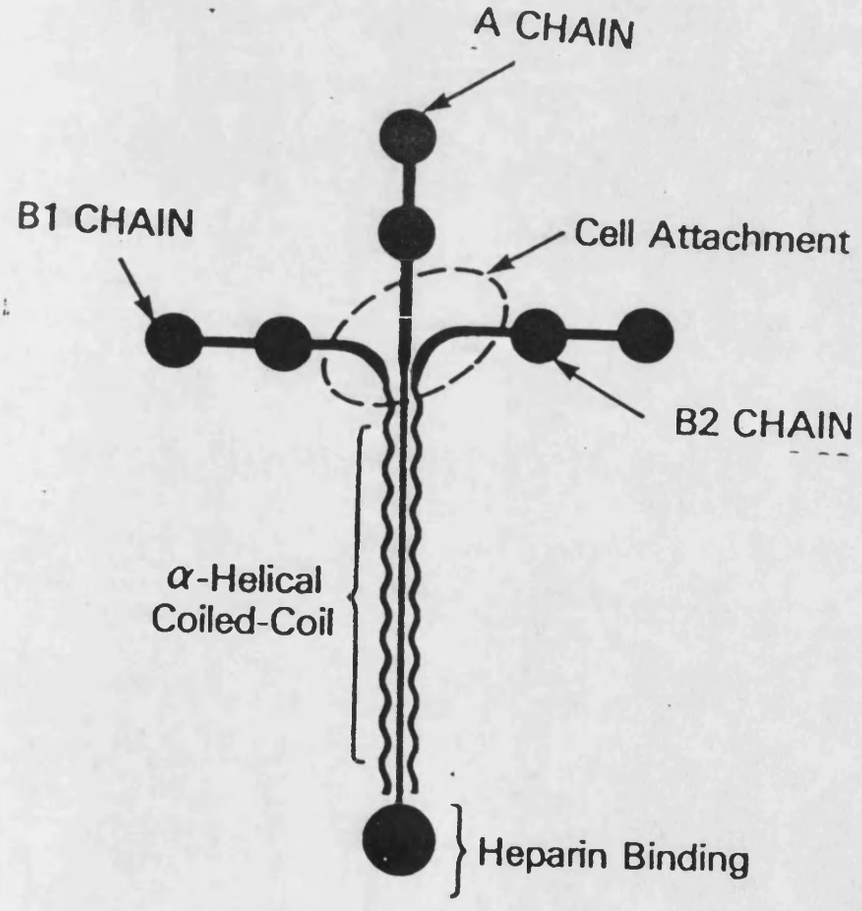


FIGURE 1.4.

A SCHEMATIC DRAWING OF THE LAMININ, WHICH SHOWS THE ARRANGEMENTS OF THE A, B1, AND B2 CHAINS WITH THE EIGHT GLOBULAR DOMAINS IN THIS CROSS SHAPED MOLECULE.

expected to be the -tyr-isoleu-gly-ser-arg- sequence, which belongs to the B1 chain of the laminin. This sequence is absent in fibronectin and other proteins which have specific sequence RGDS (Graf et al, 1987a and Iwamoto et al, 1987).

Two different peptide sequences of E8 fragments have been characterized for their capacities to promote cell attachment: one is situated near the carboxyl terminal end of the B2 chain (Sasaki & Yamada, 1987 and Liesi et al, 1989) and the other at the carboxyl terminal of the A chain (Sasaki et al, 1988 and Tashiro et al, 1989). Another site on the A chain contains the sequence -arg-gly-asp-(RGD) and is expected to be biologically active (Humphries et al, 1986 and Ruoslahti & Pierschbacher, 1987). Grant et al, (1989) suggested that peptide YIGSR acts in concert with RGD sequence of laminin A chain. The RGD peptide may mediate its cellular effects by binding with integrin receptors (membrane integrated proteins, a superfamily of receptors). However, cell attachment to laminin mediated by receptors of the integrin family is not systematically inhibited by the -arg-gly-asp-(RGD) peptide (Gehlsen et al, 1988).

Laminin at 5µg/ml promotes the attachment of various epithelial cells to plastic or to type (iv) collagen coated substrates (Graf et al, 1987a). Both fibronectin and laminin appear to be able to bind to collagen, the latter is a member of the family of extracellular matrix proteins, which have the ability to mediate the attachment and spreading of many cell types.

A conclusion on protein adsorption can be made, that is, if a culture vessel is placed in a growth medium containing serum, proteins adsorb non specifically to the surface and mediate cell adhesion. If the cells are seeded on a substrate in the absence of adsorbed proteins, then the proteins on the cell surface may directly adsorb to the surface and the cell will, providing the conditions are favourable, secrete its own proteins (e.g. fibronectin, vitronectin, laminin and collagen) toward the surface in the form of an extracellular matrix. If the substrate does not support protein adsorption, or even if it does, but there are no receptors on the cell surface, the substrate will not support cell adhesion. After protein adsorption the next cellular event in the sequence

involved in cell adhesion is the attachment of the cell to the surface. This is discussed below:-

1.2.2. CELL ATTACHMENT

As a first step towards attachment the cell makes contact with the protein coated substratum. If the cell is small, as in the case of bacteria, charges will take the cell to the surface. With larger mammalian cells gravitational forces assume the control role in cell-substrate contact (figure 1.5) (Grinnell, 1978). Following contact of the cells with the substrate, attachment bonds between cell and previously adsorbed proteins on the substratum are formed (figure 1.5) (Lydon & Foulger, 1988 and Klein-Soyer et al, 1989). One of the major challenges of cell biology and biochemistry is the elucidation of the macromolecular structures involved in attachment bonds and then to understand their functions and regulation.

As stated earlier, it is only within the last decade that many extracellular adhesion proteins such as fibronectin, laminin and vitronectin have been identified and fairly characterized. However, understanding of the binding between these extracellular matrix and cells was in a rather confused state for some time. However recent detailed analysis of the cell binding site on fibronectin led to a precise understanding of the mechanism of cell-extracellular matrix interactions. In a splendid series of studies Pierschbacher et al, 1982) used proteolytic techniques to produce successively smaller fragments of the fibronectin molecule, which retained cell adhesion promoting activity. Later they synthesized a series of peptides which overlapped the cell binding site and deduced that the critical feature of this site was a tripeptide with the sequence arg-gly-asp- (RGD) (Pierschbacher & Ruoslahti 1984).

Identification of RGD sequence was the reward of extensive efforts to define the structural basis for the adhesive properties of fibronectin. Moreover, the success in delineating the function of RGD sequence encouraged further work to similar sites within other matrix molecules (Horwitz et al, 1985; Ruoslahti & Pierschbacher, 1986; Graf et al, 1987b). The assignment of the cell binding activity of the fibronectin

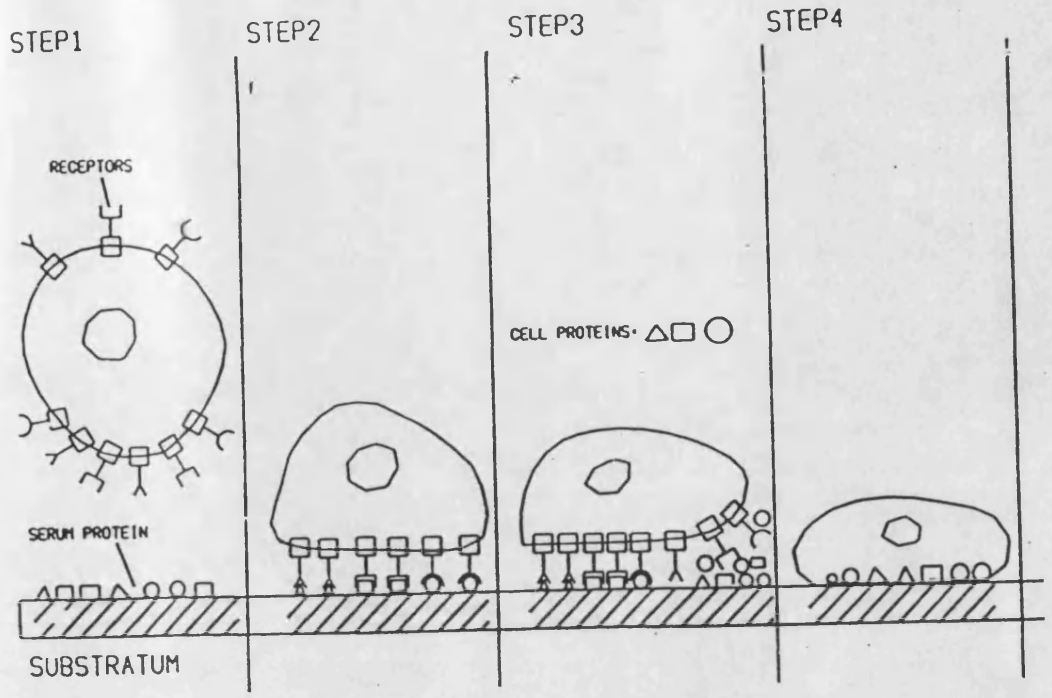


FIGURE 1.5.

SCHEMATIC ILLUSTRATION OF AN ADHERING AND SPREADING CELL IN THE PRESENCE OF SERUM PROTEINS.

STEP 1. SUSPENDED CELL REACHING THE ADSORBED SERUM PROTEINS.

STEP 2. INITIAL CONTACT OF THE CELL WITH THE ADSORBED PROTEINS AND FORMATION OF THE RECEPTOR-PROTEIN BONDS.

STEP 3. CELL-SUBSTRATE ATTACHMENT, PROTEIN SYNTHESIS AND SECRETION

STEP 4. SPREADING, FORMATION OF ADHESION PLAQUES AND GROWTH.

molecule to a tripeptide sequence(RGD) provided a strong hint that interaction between fibronectin and the cell membrane was indeed very specific and it likely involved a membrane receptor protein. This has since been confirmed by several lines of evidence (Liotta et al, 1985; Pytela et al, 1985b; Kitten et al, 1986). The cellular recognition system that comprises the receptors are collectively called integrin receptors and are briefly discussed below

1.2.2.1. INTEGRINS

The information that a cell receives from the extracellular matrix has a marked influence on its behaviour with regard to the cell adhesion. The cell surface proteins that interact with the components of the extracellular matrix are the integrin receptors. This name was coined because they play a bridging role between cytoskeleton and the extracellular matrix (figure 1.6).

Early work on adhesion receptors was done by generating antibodies to cell surface proteins that could block cell-substrate adhesion (Damsky et al, 1981; Neff et al, 1982). The results suggested that a group of proteins with molecular weights in the range of 100-150 KDa were responsible for the attachment of cells to substrates. However, the complexity of the proteins recognised by the antibodies and the ability of the antibodies to inhibit adhesion to multiple matrix proteins were confusing. Now its known that the complexity of the proteins immunoprecipitated by the adhesion inhibiting antibodies was due to the sharing of subunits among the adhesion receptors. Some years ago affinity chromatography of cell and tissue extracts on immobilized fibronectin cell attachment fragments or RGD peptide was applied to the isolation of adhesion receptors. The fibronectin fragment affinity matrix yielded, upon elution with the RGD peptide, a fibronectin receptor that consists of two polypeptides, an α and a β subunit (Pytela et al, 1985b). Affinity chromatography on vitronectin gave a vitronectin receptor that was also a heterodimer , but it consists of different sub-units

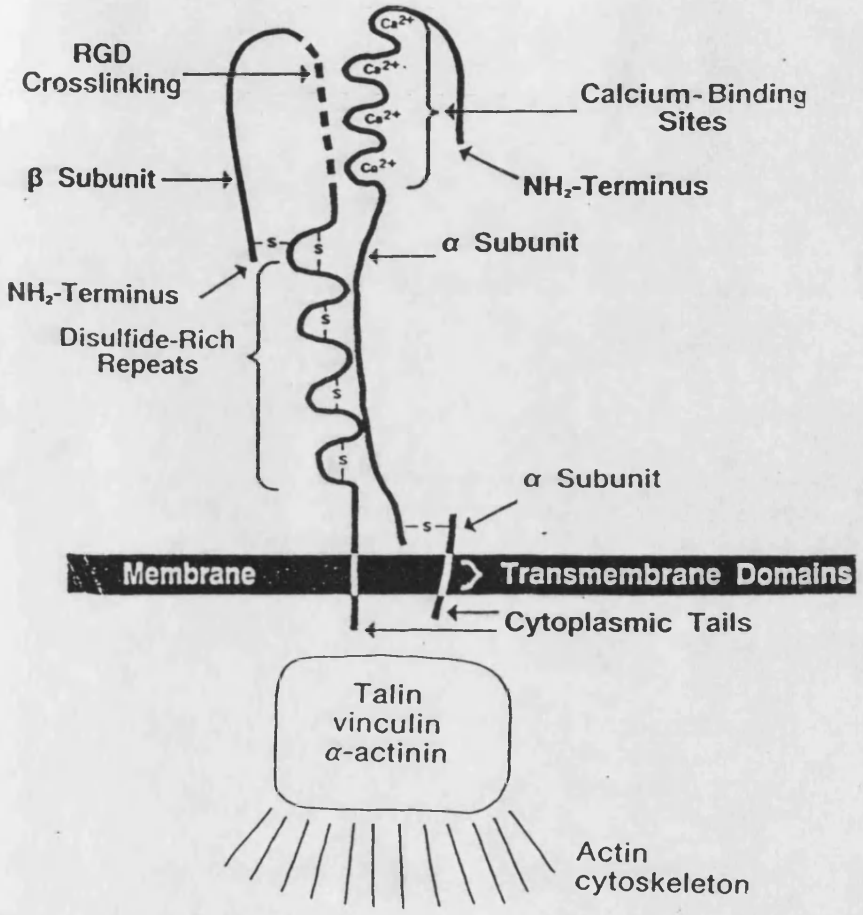


FIGURE 1.6

SCHEMATIC STRUCTURE OF A TYPICAL INTEGRIN.

than the fibronectin receptor (Pytela et al, 1985b). Both receptors could be isolated from the same osteosarcoma cells and fibroblasts. The related structures and specificities of the fibronectin and vitronectin receptors made scientists realize at that time that they were dealing with a recognition system, not just receptors to individual matrix protein. When the affinity chromatography isolation procedures were applied to other cell types, additional receptors were obtained.(Pytela et al, 1986). Subsequently these receptors were characterized and it was found that all integrins are heterodimers of non-covalently linked sub units (Hemler, 1990 and Ruoslahti, 1991).

Both subunits are integral membrane glycoproteins. Both α and β subunits of integrins have a segment with the characteristics of a transmembrane domain near the COOH terminus, suggesting that each subunit spans the membrane and that the short portion of the polypeptide extending from the COOH terminal end of the transmembrane domains is cytoplasmic. The α sub unit contains areas thought to bind calcium and the β subunit contains 4 cystine rich repeats (Marlin & Springer, 1987; Dransfield & Hogg, 1989; Kirchhofer et al, 1991). The extracellular domains contain the adhesion protein binding region. The cytoplasmic domains of both subunits are relatively small and contain regions capable of binding to cytoskeletal elements (e.g. talin, vinculine) that link the integrins to the actin cytoskeleton (Akiyama et al, 1990b). More than eleven distinct α subunits have been described. They are divided into three main sub-classes based on the β subunits, with which α chains associate. The $\beta 1$ sub family includes receptors for fibronectin, laminin and various collagen. The $\beta 2$ sub family consists of leucocyte specific receptors, while the $\beta 3$ sub family consists of platelet glycoproteins and vitronectin receptors (Kelly et al, 1987; Vogel et al, 1990; Ruoslahti, 1991).

These groupings should be considered provisional as new information concerning α/β subunits and substratum specificity is appearing at a rapid pace. The amino acid sequences of several integrins have been determined for cDNA. One of the chicken integrin complex was the first subunit sequenced (Tamkun et al, 1986). Later both subunits of the human fibronectin receptor were sequenced (Argraves et al,

1987). Now the sequence of many integrins is known. There is no sequence similarity between the α and β subunits of any one of the individual integrins but each α subunit is similar to the other α subunits and each β subunit to the other β subunits. The extent of this similarity is 40-50% at the amino acid level. The receptor binding requires divalent cations such as Ca^{2+}, Mg^{2+}, or Mn^{2+} and perhaps cations can modify the affinity and specificity of the receptors. Integrins are a multigene family and consists of many members. In this work only fibronectin receptors, vitronectin receptors and laminin receptors will be described.

1.2.2.1.1. FIBRONECTIN RECEPTORS (FNR)

Members of this family of integrins contain one of six (or more) unique but related α subunits associated with the common β subunit in a non-covalent dimer (Hemler et al, 1989). The larger component termed the α subunit usually ranges in size from 140-165Kd and the smaller β subunit is 120-130Kd in size (Argraves et al, 1987; Hemler et al, 1987; Takada et al, 1987a). The avian FNR is called α3β1 and mammalian fibroblast FNR is designated as α5β1 (Takada et al, 1987b). Current evidence suggests the avian fibronectin receptor is a multifunctional receptor or a mixture of related receptors. Monoclonal antibodies that bind to the receptor inhibit cellular interactions with fibronectin, laminin vitronectin and collagen (Buck & Horwitz, 1987). Thus at present, it is probably prudent to refer to this complex rather than simply the fibronectin receptor.

The avian integrin receptor complex also binds to the cytoskeletal protein talin but not to α-actinin or vinculine.(Horwitz et al 1986). The binding site for talin on the receptor complex is on the intracellular portion, distinct from that for fibronectin. The mammalian fibronectin receptor (α5β1) consists of a 145-155 KDa α and 125 KDa β subunit. Both subunits have been cloned and sequenced. The function of the high affinity α5β1 FNR has recently been probed using monoclonal antibodies. Although capable of mediating initial attachment of normal fibroblasts, the receptor is not essential for subsequent cell spreading on a fibronectin substratum (Akiyama et al,

1989). This function might be performed by vitronectin receptors or a recently discovered fibronectin receptor a4b1 which recognise -leu-asp-val- (LDV) sequence. (Komoriya et al, 1991).

Akiyama et al (1989) also discovered that the formation of specialized cell contacts with the substratum and actin microfilaments orgnisation seem to be partially dependent on this receptor function. The localization pattern of fibronectin receptors in the plasma membrane of cultured cells is consistent with receptor involvement in cell adhesion. On stationary cells, the receptor is organised into immobile aggregates that appear to be associated with the ends of extracellular fibronectin fibrils (Ruoslahti & Pierschbacher, 1987). Receptor function is maintained after treating cells with proteinases in the presence of Ca^{2+}, whereas receptor function is lost after treating cells in the absence of Ca^{2+}. Clearly, the fibronectin receptor is at least stabilized or protected by Ca^{2+} (Akiyama and Yamada (1985). The β1 sub family have another group of receptors which recognise laminin as described below.

1.2.2.1.2. LAMININ RECEPTORS (LMR)

Cells can interact with laminin through a multiple receptor system. Probably a mixture of β1 integrin receptors can interact with laminin, although with lower affinity than the fibronectin receptor (Horwitz et al, 1985 and Cohen et al, 1987). The α3β1 fibronectin receptor and α6β1 human platelet receptors also bind with RGD sequence within the laminin molecule (Aumailley et al, 1987, and Goodman et al, 1987). Other laminin receptors isolated from rat Rugli cells and neuronal cells (Gehlsen et al, 1988) are also β1 integrins, probably the homologs of α3β1 or α6β1. There is another laminin receptor of molecular weight 69KDa (yet to be named). The anti-69KDa antibodies inhibit cell attachment on laminin, while having no effect on fibronectin (Wewer et al, 1987). Synthetic peptide containing the sequence Tyr-Ile-Gly-Ser-Arg (YIGSR) can elute the 69 KDa receptor from laminin affinity columns (Graf et al, 1987b).

A major laminin binding protein of 56 KDa characteristic of muscle cells has been isolated and cloned from chick and rat skeletal muscle. This extracellular, membrane protein interacts with the major heparin binding domain in the laminin α1 chain (Clegg et al, 1988). There are many other LMRs yet to be named depending on whether or not the present integrin classification remain intact in the future (Sonenberg et al, (1988).

1.2.2.1.3. VITRONECTIN RECEPTORS (VNR)

The vitronectin receptors belong to the β3 sub family and they recognise RGD sequence (Pytela et al, 1986). Surprisingly, the VNR binds to vitronectin but not to fibronectin (Pytela et al, 1987). In fact VNR binds to the RGD sequence with higher affinity than the FNR (Pytela et al, 1987). It consists of a 125Kd α chain and 115d β3 chain. The localization of the VNR on cultured cells suggests that it plays a role in cell adhesion (Cheresh et al, 1987).Moreover the VNR is involved in spreading of the cells, as their association with the cytoskeleton was observed at the late stage of the cell adhesion (Singer et al, 1988). A novel vitronectin receptor (αvβ3) has been identified recently on carcinoma and other epithelial cells, which consists of the same α-subunit associated with a new β-subunit distinct from any known integrin (Hemler et al, 1989).

In conclusion, almost all integrins recognise the RGD sequence but with a different specificity for each individual protein. At the present time it is not clear how the receptors can show such specificity for individual protein(s). Two possible explanations for this can be given.

1 It could be that while the RGD sequence is critical for the receptor-ligand interaction, other sites in the ligand protein are needed to stabilize the interaction and it is these latter sites that decide the specificity

2 The other possibility is that the RGD sequence itself assumes different conformations in different proteins and it is these unique conformations that are selectively recognised. Experiments in vitro with short peptide containing RGD may

not be giving a clear picture, because short peptides are obviously more flexible than the same sequence in a protein. Thus the model peptides are capable of assuming very many different conformations, which may recognise more than one receptor.

Nevertheless, once an interaction between extracellular protein and the integrin receptor has occurred the cell attachment to the surface has been initiated. Further attachment sites develop through a state of attachment called spreading. The process of spreading and strengthening of cell adhesion requires metabolic energy (section 1.3.1). Spreading also involves biochemical and cytoskeletal events which are indicated as below.

1.2.3. SPREADING

Cell spreading begins well after initial attachment and continues during and after the first hour of initial attachment (Pethica et al, 1984 and Vogler & Bussian, 1987). The cell spreading is quite different from the initial previous steps which led to a stabilized adhesion. In order to spread (specific morphology) cells require a suitable stimulus and this is usually supplied by the serum present in the growth medium or the cell itself produces fibronectin and vitronectin like proteins (Knox & Griffith, 1980).

Horwitz et al, (1986) and Tamkun et al,(1986) suggested that spreading is mediated by an interplay between the cytoskeleton, integrins and extracellular matrix proteins. Indeed it has been known for some time that these complexes are closely localized in cell to surface contact area (Yamada, 1983 and Geiger, 1983).

For example fibronectin was found in the vicinity of focal contact sites where microfilament bundles terminate and where the plasma membrane is close to the substratum. Other proteins which are enriched in the close cell-substrate contact area (focal contact) are cytoskeletal proteins. These include: vinculine talin and α-actinin

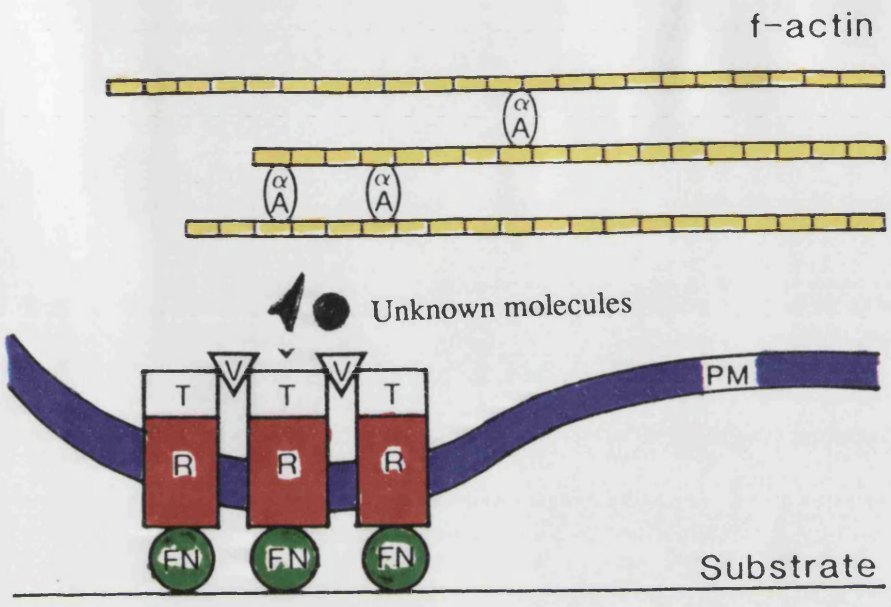


FIGURE 1.7

SCHEMATIC REPRESENTATION OF SOME OF THE COMPONENTS

LOCALIZED AT SITES OF CLOSE CELL TO SUBSTRATE ADHESION

PM = Plasma membrane
FN = Fibronectin
R = Receptor
T = Talin
V = Vinculin
α.A= α-actinin

(figure 1.7) (Burridge & Feramisco, 1980; Burridge & Mangeat, 1984; Bretscher & Weber, 1983.). Why the focal contacts form during the spreading is not clear. However, arrangements of proteins mentioned above, in a sequence on these sites indicates that spreading is an active process. This occurs via the extracellular protein-receptor-cytotoskeletal interactions in the focal contact area (figure 1.7).

The cellular mechanism controlling spreading by means of specific structures and their interactions with the cytoskeleton are still very poorly understood. So far the only evidence available is that the fibronectin receptor may directly interact with talin and vinculine of the cytoskeletal proteins (Horwitz et al, 1986).

Chen et al,(1986), Dejana et al,(1988) and Mullar et al,(1989) presented evidence that a specific ligand is required for clustering of individual receptors such as fibronectin from extracellular matrix and talin from the cytoplasmic side. They further explored the initial 30 minutes of cell adhesion where most of the cells remain round and adhere only at their periphery. At this stage fibronectin receptors are scattered all around the cell surface and just a few receptor clusters were localized in correspondence to vinculin. From thirty to sixty minutes cells flatten considerably. Receptors are scattered within the plane of ventral membrane and still do not correspond to microfilament bundles. After one hour of seeding the cells , the correspondence between receptor clusters and vinculin was considerably improved but was still far from completion. Two hours after seeding, cells acquired full flatness. At this time complete correspondence of receptors with vinculin was seen.

Muller et al, (1989) suggested that talin aggregation with fibronectin receptor may be the first events induced by fibronectin substratum, while α-actinin and actin become localized at the attachment site during the most advanced stages of spreading. Despite these interesting observations, there is little direct biochemical evidence showing that the molecules mentioned above are the only elements involved in spreading. There may be some other molecules and metabolic events participating in this complex process. which is yet to be determined. Cellular spreading is always preceded by the

process of strengthening or consolidation of cellular adhesion and it is also possibly triggered by metabolic events (section) as indicated below.

1.2.4. STRENGTHENING OF CELL ADHESION AND GROWTH OF CELLS

At this stage cells adhere tightly to the underlying substrate through discrete regions of plasma membrane, referred to as adhesion plaques (Woods & Couchman, 1988). The assembly and disassembly of adhesion plaques can be explained as below.

1.2.4.1. ADHESION PLAQUES

Many cells, including fibroblasts and epithelial cells form adhesion plaques when plated onto appropriate substrata. Adhesion plaques are generally absent in migrating cells, while they are abundant in stationary anchorage dependent cells (Kolega et al, 1982). The formation of the focal adhesion is preceded by a structural precursor consisting of a bundle of actin filaments oriented radially within the leading edge of the cell (Gieger et al, 1984 and Rinnerthaller et al, 1988). The precursor can be divided into two portions: a proximal part that becomes stationary and forms the plaque on the cytoplasmic face of the focal adhesion membrane and a distal part, which continues to advance with the leading edge (Depasquale & Izzard, 1987).

The components taking part in the formation of adhesion plaques come from three regions of the cell:

(1) extracellular face

(2) cytoskeletal face

(3) membrane components.

The extracellular face contributes, fibronectin, laminin and vitronectin (Laterra et al, 1983 and Woods et al, 1986). Actin, vinculin, talin are structural proteins of the cytoplasmic face which participate in adhesion plaques (Kupfer et al, 1986; Volberg et al, 1986 and Marchisio et al, 1987). Integrin receptors are an obvious choice for the linkage between the cytoskeleton and the extracellular matrix (Kelly et al, 1987 and

Singer et al, 1988). For example the transmembrane linkage of talin to the extracellular matrix via the fibronectin receptors provides one mechanism by which cytoplasmic components can become coupled to the extracellular matrix (Horwitz et al, 1986).

Adhesion plaques are dynamic structures, thus in cultured cells they assemble, disassemble and then reassemble at specific times during the cell growth. (Gieger et al, 1984b; Small & Rinnerthaler, 1985 and Burn et al, 1988). It is not clear how this complex assembly/disassembly mechanism is regulated. However this mechanism is attributed to limited proteolysis and phosphorylation of the extracellular matrix and cytoskeletal protein(s) (Anteler et al, 1985; Kamps et al, 1986 and Herman et al, 1986).

Giving the example of proteolysis, talin was found to be a preferred substrate for a calcium dependent protease *in vitro* (Burridge, 1986). Plasmin was found to degrade extracellular matrix e.g. fibronectin. Thus the link(s) between fibronectin, the integrin and talin can be abolished (Chen et al, 1985 and Fairbairn et al, 1985). Once this linkage is eliminated, the adhesion plaque would be disassembled.

Evidence suggesting that the phosphorylation may be another important factor in the disruption of focal adhesion in the cells has been obtained by using chemical activators of specific kinases and by transforming cells with Rous sarcoma virus. For example tumour promoting phorbol esters (TPA) induce a rapid loss of stress fibres and displacement of vinculine from adhesion plaques (Kellie et al 1985, Meigs and Wang, 1986, Lamb et al, 1988).

Recently, biochemical studies have shown that Rous sarcoma virus(RSV)-kinase is found tightly associated with plasma membrane of RSV transformed cells. The above mentioned kinase was able to phosphorylate a member of the integrin family i.e. the fibronectin receptor. It was further noticed that the phosphorylation of these fibronectin receptors also uncouples the linkage of talin with the extracellular matrix, thereby disassembling the adhesion plaques. Adhesion plaques are thought to be structures essential for growth of cells.

These observations (referred to above) suggest that the extracellular matrix macromolecules, presumably sending some informations to the cell interior by acting through their membrane receptors, can modulate the growth of the cells. A brief insight into thiis aspect of adhesion is given below.

1.2.4.2. PROTEIN-RECEPTOR INTERACTION; "THE REGULATOR OF CELL GROWTH"

The mechanism by which the control of cell growth might be accomplished is still sketchy. However, recently considerable progress has been made toward the understanding of this rather sophisticated mechanism. It was known for some time that the composition of the extracellular matrix-cell surface complex had important regulatory and structural consequences for the cells. For example , the interaction of a cell with either of two purified matrix molecules e.g. collagen and fibronectin, can have profoundly different adhesion, biosynthetic pattern and capacity for that cell to migrate and proliferate. (Yamada, 1983).

A striking change in morphology was observed when fully differentiated epithelial cells were cultured on purified collagen. The cells underwent a fundamental alteration in morphology from epithelial to mesenchymal cells.(Greenberg & Hay, 1982). Yamada et al (1976) treated oncogenically transformed cells, originally, deficient in fibronectin, with purified exogenous cellular fibronectin. They found that the transformed cells were restored to a normal cell shape, normal adhesiveness and actin microfilament orgnisation. Furthermore, extracellular matrix molecules can also regulate the amount and type of the other extracellular molecules (Klienman et al, 1981). For example the fibronectin could considerably stimulate the synthesis of collagen by hepatocytes (Foidrt et al, 1980, and Klienman, 1981).

Recently, one hypothesis put forward for the control of cell growth is that extracellular proteins through their binding with the cytoskeleton via their receptors, send direct signals to the interior of the cells and control its growth. (Unemori and Werb, 1986). It is difficult to visualise how the binding of an extracellular molecule to

a transmembrane receptor can produce changes in cell behaviour. A solution to this puzzle is just beginning to emerge. For example, Menko and Boettiger, (1987) suggested that binding of extracellular molecule(s) to integrin (s) may directly activate a second messenger system(s) and initiate a signal that is then transduced to the nucleus to influence gene expression and cell growth.

The various stages in cell adhesion have been described. It is now appropriate to discuss some of the factors which effect the cell adhesion. These factors include:

1- Energy and protein synthesis (active or passive adhesion)

2- Inducers of cell adhesion:

(i) divalent cations

(ii) others

1.3.IS CELL ADHESION AN ACTIVE OR PASSIVE PROCESS ?

The facts which are given in above sections and other lines of evidence (including present work) suggest that cell adhesion is a metabolically active process. Unfortunately, there is no consensus on this issue, rather conflicting reports have been published now for more than two decades. A brief review of this long debated issue will be stated as below.

1.3.1. METABOLIC ENERGY

Carter (1967) suggested that spreading of mouse fibroblasts on cellulose acetate sheet was passive. The passive spreading means that spreading is not the result of the forces or components which originated from inside the cell, rather it is due to forces acting between the surface of the cell and the surface of the substrate.

Wolpert et al (1969) rejected the idea of passive spreading by saying that if cell spreading was caused by passive process, it ought not be significantly affected by lower temperature, which did however reduce active cell attachment. To prove that adhesion is an active process Michaelis and Dalgrano (1971) were able to inhibit the attachment of pig kidney cells to glass by using metabolic inhibitors. However they

were convinced that energy is required for the molecular conformational changes in the cell periphery but not for the *de novo* synthesis of new proteins (this protein issue will be addressed shortly).

Klebe (1975) went one step further. He blocked ATP synthesis with metabolic inhibitors and was able to show inhibition of cell attachment. Metabolically inhibited but viable cells regained their ability to attach to the substratum, when glucose was added to the medium, while metabolically dead fibroblast cells did not attach to the substrate. In contrary findings to these observations Nath & Srere (1977) demonstrated that the inhibition of ATP synthesis was not important in cell adhesion. Recently, Maruyama et al (1989) investigated the role of cellular metabolism in the lymphocyte adsorption on poly hydroxy methyl methacrilate (poly HEMA) and polyamine co- polymers. They deliberately varied the metabolic process either by lowering temperature ($4^{o}c$) or by treatment with colchicine that disrupts the structure of cytoskeleton.

In response to low temperature and colchicine, the cell attachment and spreading were considerably inhibited on poly HEMA while these processes remained non responsive on polyamine co- polymer surfaces. In fact they left the debate about the involvement of energy in cell adhesion open to several interpretations.

1.3.2. REQUIREMENT FOR PROTEIN SYNTHESIS

If the cell adhesion is an active process along with an energy need, the protein requirements for cell adhesion should be a necessity. Against this notion, the role of protein synthesis in cell adhesion has been always controversial. For example as stated earlier, Michaelis and Delgrano (1971) proposed that energy is one requirement of cell adhesion while protein synthesis is not relevant to cell adhesion. Indeed, to date several contradictory reports have been published. The most important of these will be considered in the following paragraphs.

Weiss and Chang (1973) published a completely conflicting report. They noted that when ascites tumor cells were deprived of protein synthesis with cycloheximide (up

to 1μg/ml) this resulted in an increased rate of cell adhesion. Although no other report in favour of their idea has come by in the literature yet, the relevance of protein synthesis in cell adhesion has become a controversial issue. Pena & Houghes (1978) demonstrated that baby hamster kidney cells (BHK) seeded on fibronectin coated surfaces acquired spreading, which was independent of cell protein or nucleic acid synthesis. From these observations, the role of protein synthesis in cell adhesion cannot be ruled out because an important member of the extracellular matrix is fibronectin, this itself suggests the possible role of protein(s) in cell adhesion.

Grinnell & Feld (1980) found that if fibronectin secretion is inhibited, cell spreading is inhibited unless the surfaces are coated with fibronectin. The ability of human fibroblasts to attach to native and denatured collagen in the presence or absence of fibronectin was studied (Farsi et al 1985). It was found that the attachment was independent of serum or exogenous fibronectin. However, the attachment in the absence of serum or fibronectin (fibronectin was blocked with antibody) was found to be dependent on cellular protein synthesis. Albelda et al (1989) seeded endothelial cells on a fibrinogen coated substratum and exposed them to cycloheximide (25μg/ml) . This cycloheximide treatment was able to eliminate most of the fibronectin fibrilar along with fibronectin receptor orgnisation. These results indicated the importance of fibronectin and endogenous protein synthesis.

Recently, Flickinger & Culp (1990) noted that on a collagen substratum prolonged cycloheximide treatment failed to reorgnise the actin into stress fibres. While on fibronectin coated substratum cycloheximide treatment had no effect on stress fibre formation. These studies clearly suggest a role for fibronectin and other endogenous proteins for successful cell adhesion. A contrary report by Neumier et al (1985) found that hepatocytes were well adhered on plastic in the presence of serum or fibronectin and subsequent spreading could not be prevented when protein synthesis was inhibited with cycloheximide (28μg/ml).

As stated earlier (section 1.2.1) a major controversy took place when Curtis et al (1983, 1984, 1986, 1987) ruled out the role of fibronectin as a mediator of cell

adhesion. Rather they suggested that fibronectin is an activator of cell adhesion. Curtis gave credit to hydroxyl groups on polymer surfaces as mediators of cell adhesion. Lydon et al (1985) criticized this idea on the basis of the facts that adhesion is inhibited on the hydroxyl rich polymer poly HEMA. Smentana et al(1990) discarded Lydon et al 's arguments in favour of Curtis and suggested that hydroxyl groups in hydrogel like poly HEMA induce adhering and spreading of the cells.
It may be a misleading debate over whether or not cell adhesion is passive or an active phenomenon. It seems more likely that where cell adhesion was considered passive, spreading in serum free medium may depend upon the active secretion of adhesion proteins to the extracellular matrix. Moreover, a number of observations (Price, 1970; Wolpert et al, 1969) including our own experience in our laboratory suggest that for a variety of cell types there is no passive spreading as cells remained rounded in serum free medium.

In evaluating the role of protein synthesis in cell adhesion it is very important to determine whether the cell adhesion occurs in the absence of endogenous protein synthesis. However simply stopping protein synthesis may not be sufficient, since cells can retain adhesion proteins as a large internal pool. The secretion of proteins from this pool may mediate adhesion. Perhaps together, inhibition of protein synthesis plus secretion in the presence of antibody to specific adhesion protein or peptide inhibitors would be an important tool to evaluate the role of protein synthesis in cell adhesion

At this point it is important to take a brief look at the inhibitors of both protein synthesis and secretion. The details of protein synthesis and secretion are available in almost every biochemistry and cell biology text book. Therefore, at present, the mode of action of only very common protein synthesis inhibitors will be briefly discussed. For example Actinomycin D binds to DNA and blocks the movement of RNA polymerase and in this way prevents RNA synthesis which results in protein synthesis inhibition. Another inhibitor that blocks protein synthesis at the transcriptional level is alpha- amantin which blocks synthesis of mRNA by binding to RNA polymerase.

Some agents inhibit protein synthesis at the elongation step e.g. Puromycin causes premature release of nascent polypeptide by its addition to growing chain end.

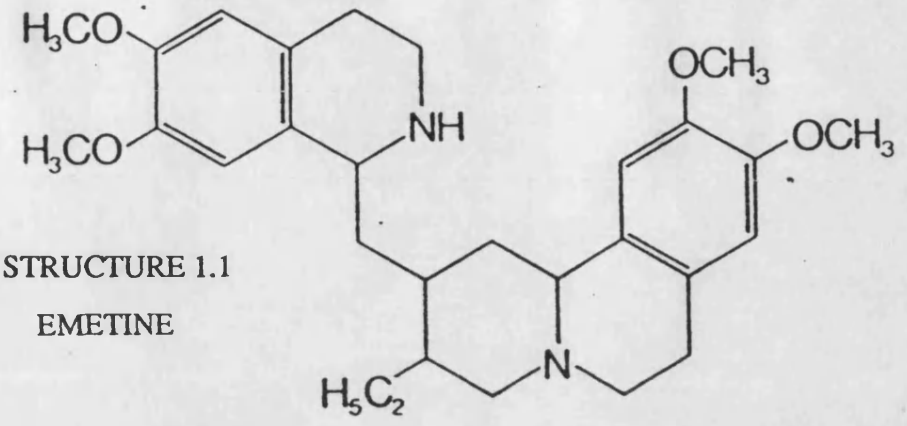
Cycloheximide and emetine are well known and effective protein synthesis inhibitors. Emetine (structure 1.1) is prepared from the ground roots of Uragoga Ipecacuhana and is the principal alkoloid of this plant. It stabilizes the 80 s eukaryotic ribosomes, so that they can no longer move along mRNA and so irreversibly inhibits protein synthesis (Olienic, 1977). Cycloheximide (structure 1.2) is obtained from cultures of *streptomyces griseus*. It blocks the translocation reaction on ribosomes, thus inhibiting chain initiation as well as chain elongation. The latter by acting on the 60 s sub unit of the eukaryotic ribosomes (Obrig et al, 1971).

Intracellular secretory pathways can be impaired by microtubule disrupting drugs such as cholchicine, vinblastin and uncouplars of oxidative phosophorylation (Kruse & Bernstein, 1975; Harwood et al, 1976; Dehan and Prockop, 1972).

The secretion of some adhesion proteins can be impaired in response to monovalent ionophores. For example monensin (structure 1.3) a monovalent ionophore can inhibit secretion of fibronectin and collagen from cultured human fibroblasts (Mollenhauer et al, 1990).

Monensin is a well characterized metabolite of *Streptomyces cinnamonnensis* that binds to ions with specificity of Ag> Na> K> Rb> Cs> Li> Ca . The binding specificity of monensin to sodium ion is ten times more than to potassium ions. As indicated in the structure 1.3 the alkyl groups are spread over the outer surface rendering the complex lipid soluble thus allowing the monensin to enter the cell membrane. It causes the exchange of protons with sodium and thus impairs the secretory pathway. (Mollenhauer et al, 1990). Apart from the inhibition, there are some inducers known to exert a wide range of biological effects on tissue culture, the most important being the induction of cell adhesion.

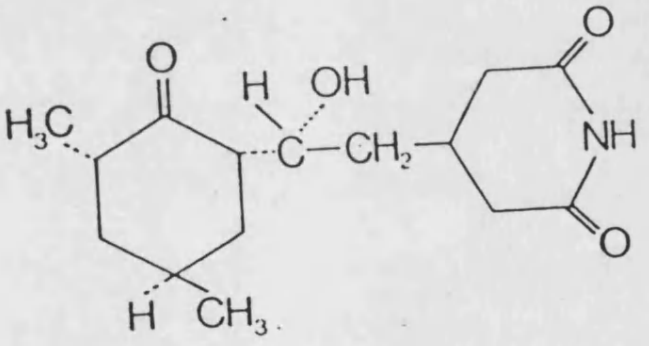
$C_{29}H_{40}N_2O_4$



STRUCTURE 1.1

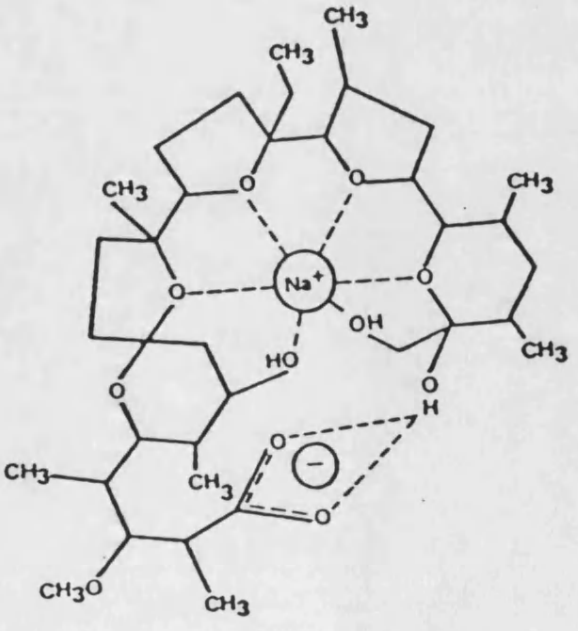
EMETINE

$C_{15}H_{23}NO_4$



STRUCTURE 1.2

CYCLOHEXIMIDE



STRUCTURE 1.3

MONENSIN

1.3.3. INDUCERS OF CELL ADHESION

These inducers include:

1- Divalent cations

2- Others (see latter)

1.3.3.1. DIVALENT CATIONS

Many lines of evidence suggest a role of divalent cation in cell adhesion. Both Mg^{2+} and Ca^{2+} appear to be active at physiological concentrations in many of the systems tested (Grinnell, 1976; Takeichi & Okada, 1972; Maroudas, 1975) observed that Mn^{2+} was able to promote the attachment and spreading of BHK cells in serum free medium. Grinnell & Lamke (1984) suggested a specific binding site for cations on the cell surface. Later Edwards et al (1987) proposed that divalent cation binding site is an extracellular site. These authors undertook a classical study and found that adhesion and spreading of BHK21 cells in the presence of serum or vitronectin require divalent cations in the order of $Mn^{2+}>Co^{2+}>Mg^{2+}>Ca^{2+}$. Surprisingly on purified fibronectin no added divalent cation was required, since the requirement was largely met by adventitious Ca^{2+}. On the basis of such a difference in divalent cation requirements between different protein binding surfaces, they pointed a site for divalent cation on the extracellular surface.

Simultaneously. Argraves et al (1987) deduced an amino acid sequence of a subunit ecto domain from the cDNA of the fibronectin receptor and found that the a-sub unit ectodomain has five sequence elements homologos to Ca^{2+}binding sites similar or homologos to several proteins like calmodulin (Szeberg et al, 1981). Later Suzuki et al(1987) and smith & Cheresh (1988) demonstrated Ca^{2+} binding site on a-sub unit of vitronectin receptor. They also discovered multiple short sequence elements that are homologos to the Ca^{2+} binding elements of other proteins, again like calmodulin. Now it has been discovered that almost every integrin receptors has a divalent cation binding site (Akiyama et al, 1990a). Becham and Jacobson (1990)

presented evidence that Mg^{2+} promotes complete Hela cell spreading on RGDS coated peptides. Biochemical evidence was presented that the interaction of collagen receptors with RGDS containing peptide is enhanced in the presence of Mg^{2+}.

After Kirchofer et al 's (1990) findings, scientists began to suspect the divalent binding site on β-sub units or the role of β-sub units in creating divalent cation site in α-sub units. Kirchofoer et al (1990) demonstrated the effect of Ca^{2+}on two different integrins i.e. one β1 and and other β3. Both of these have different responses to Ca^{2+}. The former was insensitive to calcium but responsive to magnesium while the latter was sensitive to calcium . Loftus et al (1990) gave a precise picture of Ca^{2+} binding to integrins. It has been proposed that the aspartic acid in the RGD is involved in coordinating a divalent ion between the protein and divalent binding site within the α and/or β subunits of the integrins. Clearly more studies are needed to establish the role of other divalent ions such as Mn^{2+} and Co^{2+}.

1.3.3.2. OTHER INDUCERS

These inducers are diverse in their nature with different effects on normal and tumor cells in culture. The most important effect being the modulation of cell adhesion. For example epidermal growth factor induced synthesis of fibronectin *via* inducing the mRNA corresponding to fibronectin. Deposition of fibronectin on the substratum was also induced in response to epidermal growth factor (Seebacher et al 1988). Similarly vitamin D3 treated cells were reported to be clearly more adherent than control cells. This effect was attributed to induction of fibronectin synthesis (Franceschi, et al, 1987).

Glass et al (1988) have demonstrated that Arg-vasopression and prostaglandin E2 were effective agents in the induction of mesenglial cell adhesion. When the fibronectin receptor was used as a model, phorbol ester treatment caused a rapid and profound enhancement of integrin mediated CHO (Chinese hamster ovary cells) cell adhesion (Danilov & Juliano, 1989). Since it has been found that fibronectin is either

totally absent or present in very small amounts in transformed and tumor cells, these inducers are beginning to make their place in chemotherapy of cancer.

The last and most important point to be made in the present work concerns how cell adhesion is determined. Cell adhesion has been observed qualitatively for many years. Unfortunately very few quantitative methods of cell adhesion measurement have been proposed. However, their limitations and accuracy never allowed them to become universally accepted cell adhesion measurement methods. Their complexity and non reproducibility is reviewed in the following section.

1.4. MEASUREMENT OF CELL ADHESION

In general, adhesion of a cell attached to the substratum is defined according to the shear force the cell must resist to avoid being dislodged. It is not necessary that attachment or detachment should be the exact reverse of each other. However both types of measurement have much in common, that is they coexist in any system in which cells are brought to the surface with the possibility of attachment. The greater the shear force in a system is used, the greater the number of bonds of adhesion required before an individual cell can be observed to be attached. Similarly, the greater the shear force used the more readily cell detachment occurs from the substratum (Bell, 1978).

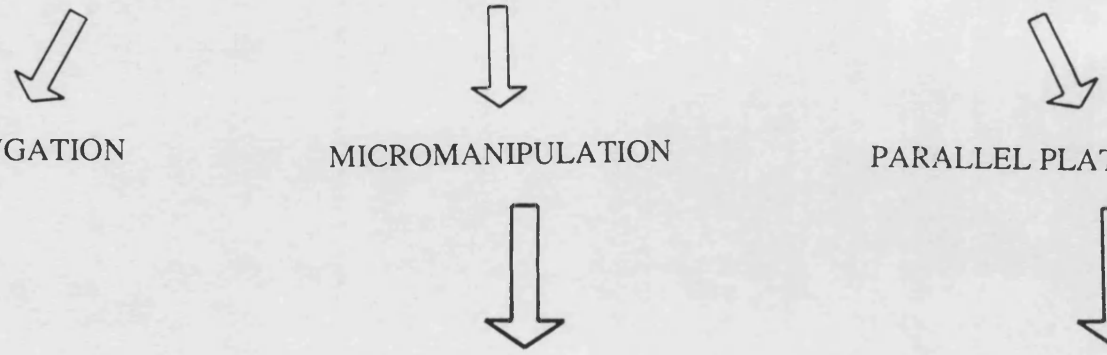
All the current methodology for the measurement of cell adhesion revolves around the principles stated above. However, there have been substantial variations from laboratory to laboratory on the kind of distractive force which is applied to a population of cells adherent to the surface. Conveniently these forces can be categorised into three main classes (figure 1.8):

1- Centrifugation

2- Micromanipulation

3-Hydrodynamic shear force

CLASSIFICATION OF THE TECHNIQUES OF MEASUREMENT OF CELL ADHESION



CENTIFUGATION	MICROMANIPULATION	PARALLEL PLATE FLOW CHAMBER
Dan (1936)	Coman (1944)	Weiss (1961)
Easty et al (1960)	Coman (1961)	Mohandas et al (1974)
McClay et al(1981)	Francis et al (1987)	Foresster and Lackie (1984)
Lotz et al(1989)	Bowers et al (1989)	Crouch et al (1985)
		Owens et al (1987)
		Doroszewski and Kiwala (1988)

FIGURE 1.8

1.4.1. CENTRIFUGATION

In this method detachment forces on cells are applied in the form of gravity or a centrifugal force. Many studies quote Dan (1936) the first person who used this technique but Easty et al (1960) appeared to develop it further. Later diverse modifications in this method have been made to measure cell-cell or cell-substratum adhesion. For example McClay et al (1981) made the cell radioactive and brought it to the surface for making contact with the monolayer of cells in a sealed compartment by a low centrifugation force. This assembly was inverted in a centrifuge and a centrifugal force applied to detach the probe cell (radioactive cell) from the monolayer. By varying the centrifugal force in term of speed of centrifuge McClay et al were able to measure attachment and detachment of chicken neural cells.

Goerge et al (1980) determined the adhesion of human erthrocytes to glass by using this centrifugal technique. A cell suspension was injected into the chamber and was allowed to settle for 10 minutes and the chambers were inverted in the centrifuge which was spun at 3000rpm. After removing the chamber, adherent and non adherent cells were counted. The centrifugal detachment force was calculated as a function of cell mass and centrifugal speed. Recently Lotz et al (1989) modified the method of McClay et al (1981). They labelled the cells with ^{3}H-leucine which were added to a plate in microtitre well. The second microtitre well was inverted over the first micrititre well and were sealed together with a gasket. The cells were attached by gentle centrifugation onto the plate in the first well. The plate is inverted immediately after gentle centrifugation and various known forces are applied to detach the cells from the plate. At this stage the top and bottoms of two microtitre wells were cut and the detachment was quantified by scintillation counting (figure 1.9).

The centrifugal method is rather uninformative. Because many cells remain adherent in response to the detachment force might represent increased adhesion or a change in cell shape which did not allow them to detach In addition, the centrifugal technique tends to be time consuming and limited in the range of forces which can be

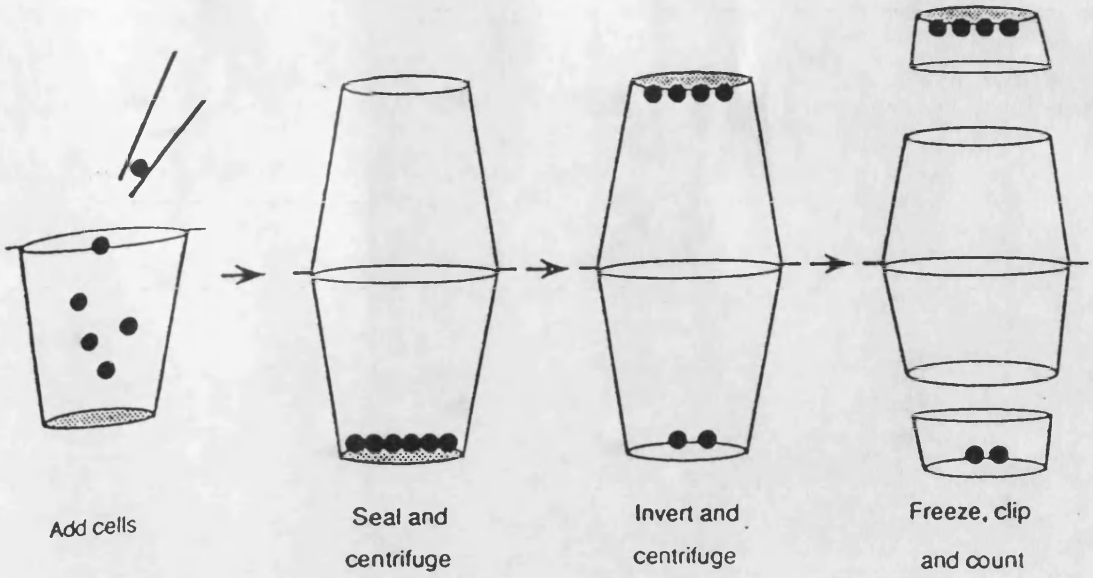


FIGURE 1.9

A CENTRIFUGAL FORCE BASED ADHESION ASSAY (Lotz et al, 1989).

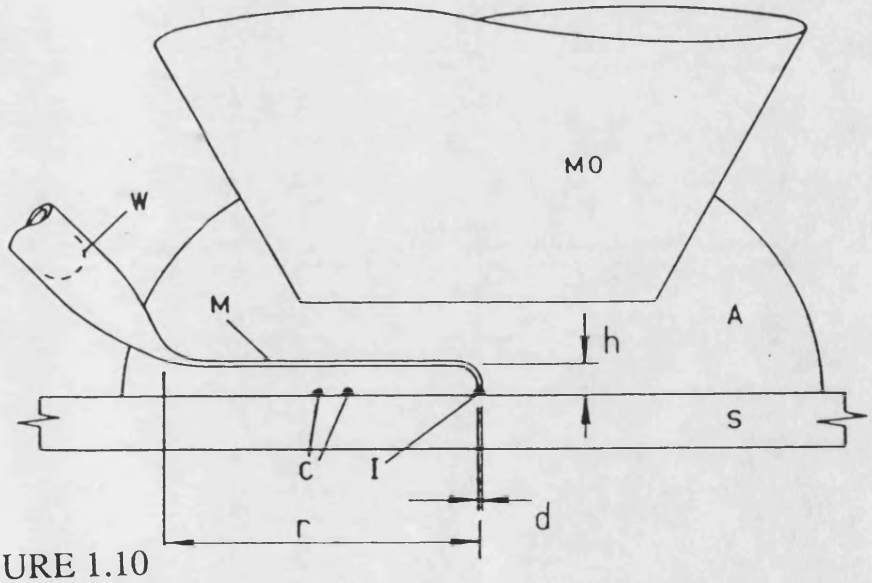


FIGURE 1.10

A MICROMANIPULATION BASED CELL ADHESION MEASURING DEVICE (Bowers et al, 1989). DIAGRAM SHOWS
M = Micrppipette
MO = Microscope objective
I = An adhering cell under investigation
C = Other cells
S = Substrate for cell attachment
A = Aqueous medium surrounding the cells.

applied to detach the fully spread cells. Often the strength of the final adhesion is so large that it exceeds, technically the ability to measure it by centrifugal method.
Few other methods have been developed by which a known force could be applied to an individual cell to break the adhesion. These methods are called Micromanipulation and are discussed below.

1.4.2. MICROMANIPULATION

In these systems the adhesion of a single cell to the substratum or separation of pairs of cells can be studied . Coman (1944) introduced this method in its simplest form. Coman (1961) used a calibrated microneedle to separate two adherent cells from each other and cell from substratum. The position of the tip of the flexible needle just prior to detachment of the cell was compared to its unstressed position when the cell to substrate contact was broken.

A few other workers have modified this method. For instance Evans (1984) developed a method on the basis of Coman's technique. He applied a force which sucked cells into a micropipette at known pressure and at the same time the shape of cells in response to suction was observed. However, the adhesion zone was not directly observed in Evan's experiment. Later, Frances et al (1987) combined the elements of Coman (1961) and Evan's methodology. Frances et al (1987) applied the force to the adherent cell via a flexible fine calibrated micropipette. The micropipette was attached to the adherent cell into a position so that it can be sucked into the micropipette. Measurement of cell adhesion can be made by calculating the applied force from the degree of bending of the pipette. The main advantage of this procedure was the direct observation of adherent zone in response to applied force. This was done by using interference reflection microscopy (specially designed for observing the contact zone). All the experimental information is recorded on a recorder.
Two years latter, a group of workers (Bower's et al, 1989) came up with a very complex new version of the Frances et al' methodology. In a prototype experimental

design the microscope plays a central role as it supports the cell attached substratum. A calibrated vertically oscillating micropipette is also positioned between the microscope and the cell bound substrate. The application of the micropipette is manipulated electrically. This assembly is attached with a pressure control system which allows the reduction in pressure resulting detachment of the cell from the surface. Measurement of the pressure and time is continuously displayed on a video screen and recorded from a video camera (figure 1.10).

Although through these refinements on Comans (1961) method avoided puncturing and tearing of a cell, how sad it is that with such a complex, expensive and high technology equipped method, only the adhesion of a single cell out of millions of cells can be measured at a time. Therefore the complexity and time consuming and the fact that relatively small number of cells can be examined in a whole day, contributes to the limitations of this procedure. The limitations of centrifugal and micromanipulated techniques could be avoided by using a hydrodynamic shear force on the cells. The brief description of these techniques is given as follow.

1.4.3. HYDRODYNAMIC SHEAR FORCE METHODS

In these kind of assays, the cells are first allowed to settle on a substrate and a hydrodynamic force is applied parallel to the surface. In attachment studies, the basic principle is almost the same in all the techniques developed for this purpose (figure 1.11). A cell containing suspension is passed over the surface and the number of cells which are settled and make contact with the surface at particular flow rate of suspension is measured after a specific time (Weiss, 1961; Mohandas, 1974; Forresster & Lackie, 1984; Doroszewski & Kiwala, 1988).

For detachment studies adherent cells are exposed to a known shear field, usually a laminar flow is claimed to be applied to the adherent cells. In these categories the simplest and easiest method is the parallel plate flow chambers. The flow through these chambers is supposed to be laminar flow (the flow rate is greatest in the centre and least adjacent to the walls).

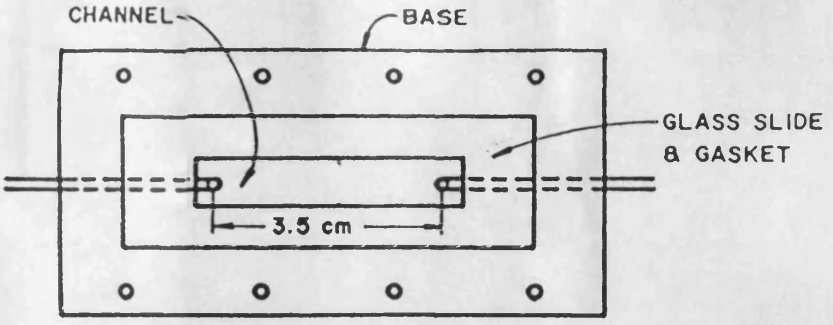


FIGURE 1.11
A SIMPLE PARALLEL PLATE FLOW CHAMBER (Mohandas et al, 1974).

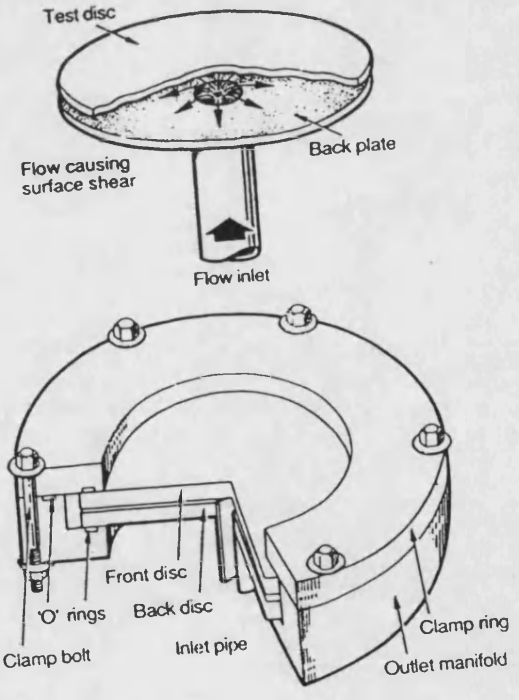


FIGURE 1.12
CROUCH ET AL`S (1985) RADIAL FLOW CHAMBER.

COMPONENTS OF THE CHAMBER

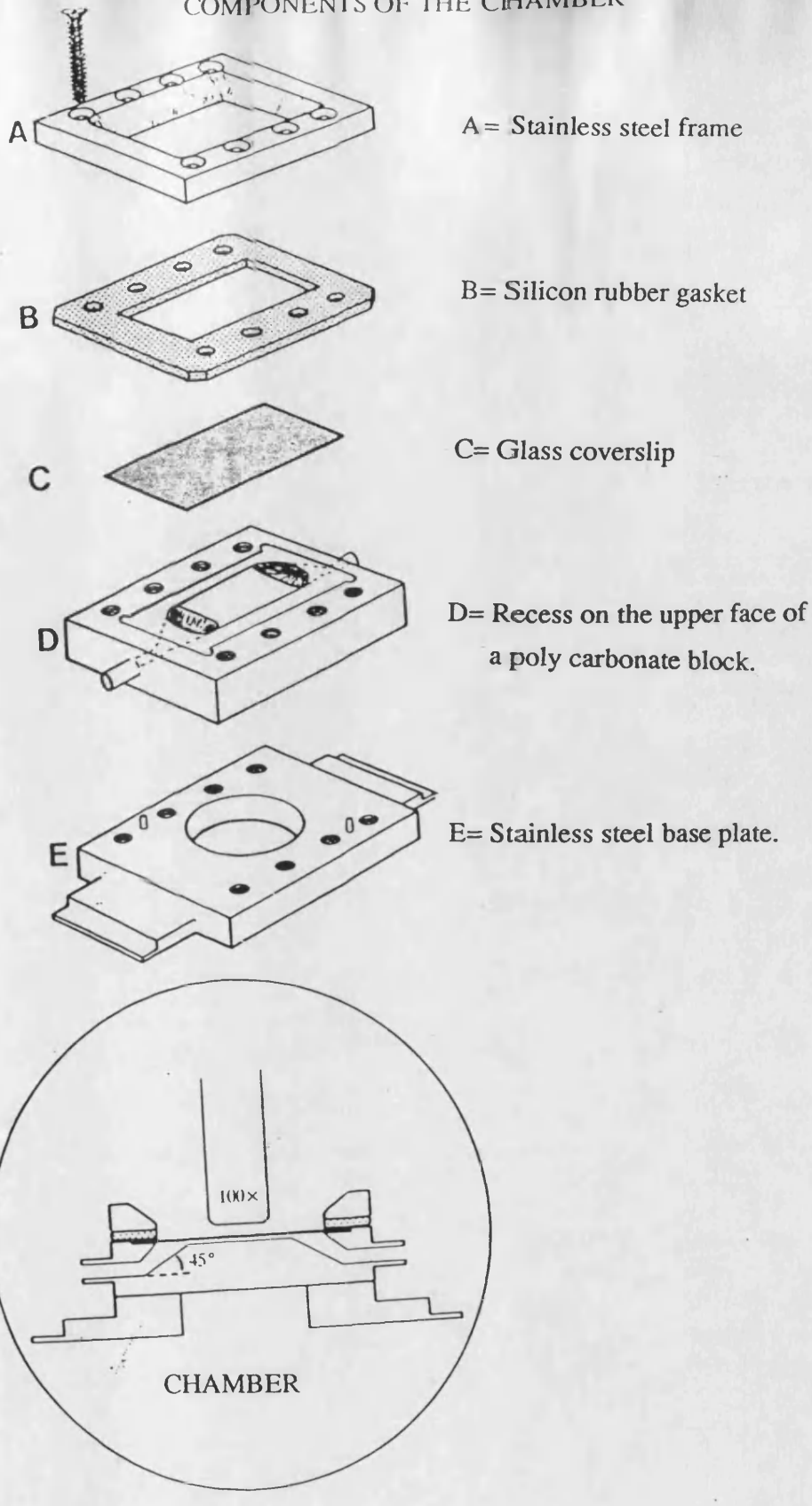


FIGURE 1.13

THE PARALLEL PLATE FLOW CHAMBER (Owens et al, 1987)

Mohandas (1974) introduced very basic design and later number of other workers used this basic design to develop their own parallel plate chambers. (Doroszewski et al, 1979; Owens et al, 1987; Trusky & Pirone, 1990).

It is also appropriate to describe the methodology of Mohandas et al (1974) (figure 1.11). In this design the upper portion of a parallel plate flow chamber contains a cell growing slide. The lower portion has a rectangular design. Fluid flows into and out of the channel through two holes drilled in the glass slides. As fluid passes from inlet toward outlet the pressure drops and number of cells which detach from the surface as a function of time and applied force are counted. The time and force are used to calculate a value below which essentially no cells will detach but above which all cells will detach, this is called minimum critical shear value.

Among the latest generation of parallel plate chambers is the one which Owens & Gingell (1987) calibrated for red cells and E. coli adhesion (figure 1.13). These workers used a laminar flow which enters the conduit through cylindrical pipe integral with the end leading into an elliptical section and then into the rectangular conduit. The flow rate through the conduit can be varied by means of a micrometer valves mounted in parallel with the flow line. The response of cells to the shear stress is recorded using a video camera. They found the range of critical shear values from 2.3 Nm^{-2} to $5Nm^{-2}$ for the red cells and *E. coli.*

The system is suitable for bacterial and red cells. It could be inefficient in measuring the adhesion of mammalian cells which adhere very tightly to the surface. Therefore, the measurement of such cell adhesion could be beyond the ability of this apparatus. Moreover the flow through the conduit is not laminar stabilized which can produce misguiding results.

The first attempt to measure the mammalian cell adhesion was by Crouch et al (1985). They developed a radial flow chamber, containing two discs separated from each other by a very small distance. The fluid is pumped into the centre of these discs. As it flows radially outwards, its velocity decreases in a way that a shear force gradient is generated (figure 1.12). The radial distance between the centre of the test

disc and edge of the zone of cell attachment can be used to determine the critical shear stress of detachment. Abnormal flow patterns were observed in this set of apparatus. It is also limited in the range of detachment forces because at higher flow rate, the stability of flow can be questioned.

In conclusion, all kind of instruments have been developed for measurement of cell adhesion. They all suffer from the need for complex equipment and are further handicapped because of limited, and indefinable ranges of detachment forces.
Having briefly reviewed the methods of measurement of cell adhesion, it was realised that a simple, reproducible and accurate instrument was required to measure the mammalian cell adhesion.
For this purpose, in the present work a simple but highly reproducible technique called a Microflow chamber has been developed (chapter 3). It is a cell adhesion measurement device in which a convergent channel has been devised. This convergent channel is the first of its kind in the field of cell adhesion in which the channel produces a complete laminar flow throught the chamber. With such laminar flow a definable force can be applied to the cell growing surface and the strength of the cell-substrate interaction can be measured in terms of a critical shear stress of detachment (chapter 3).
The present work has also as its objectives to understand the underlying mechanism of the adhesion strengthening phenomenon. In this respect, the adhesion strength of different mammalian cells has been measured and the possible mechanisms involved in this process discussed. Moreover the action of some major factors have been implicated allowing us to explore the complexity of the adhesion strengthening phenomena. Among these factors, the role of serum (different concentrations and origin), adhesion proteins (fibronectin & laminin), the recognition sequences of fibronectin and laminin, protein synthesis and secretion on adhesion strengthening phenomena are topics of the following chapters.

CHAPTER 2
MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. GENERAL

All chemicals used in the preparation of solutions were from Sigma (London), Poole, Dorset, BDH Chemical LTD. , Poole, Dorset, Aldrich Chemical Company, Gillingham, Dorset, Flow Laboratories, Irvin, Scotland and Fission Scientific Apparatus England.

All tissue culture flasks and dishes were from Sterilin LTD. U.K.

2.1.2. CELL LINES

The cell lines tested in present work include: BHK21 (baby hamster kidney cells), L929 (NCTC clone) (mouse fibroblasts) , CHL (Chinese hamster lung fibroblasts), 3T3 (Swiss albino mouse embryo fibroblasts), Walker rat carcinoma cells, Hela B (human cervical carcinoma epithelial cells) and MDCK (Madine Darby canine kidney epithelial cells). All these cell types were obtained from Flow Laboratories.

2.1.3. CELL CULTURE

PBS (phosphate buffer saline), L-Glutamine, penicillin-streptomycin, non essential amino acids, 10x minimum essential medium Eagles (modified with earls salt, 10x RPMI 1640 medium without sodium bicarbonate and Trypan blue (0.4% w/v) in 0.85% saline solution were purchased from Flow laboratories. DMEM (Dulbecco's modification of Eagles medium was from Gibco Europe LTD. HEPES(N-2-hydroxyethyl piperazine- N-2-ethanesulphonic acid was from BDH.

Trypsin and trypsin inhibitor(soybean trypsin inhibitor) was from Sigma. Nutidoma S.P. (serum free media was obtained from Boehringer Mannheim Biochemica.

2.1.4. SERA

Heat inactivated donor horse and donor calf serum were obtained from Flow Laboratories. Heat inactivated foetal calf serum was from Globepharm limited Surrey, U.K.

2.1.5. MICROFLOW CHAMBER

Peristaltic pump was from Watson Marlowe LTD. Glass slides were purchased from Chance Proper Limited. Square plastic dishes (144 cm^2) were from Sarstetd Ltd. Beaumont Leys, Leicester, U.K. and tissue culture grade round petri dishes were obtained from Sterilin Limited U.K.

2.1.6. MODIFICATION OF SURFACES

Human plasma fibronectin and ^{125}I-fibronectin (human plasma) were from Flow Laboratories and laminin (Englebreth Holm- Swarm Mouse Sarcoma) was from Sigma.

2.1.7. PEPTIDES

RGDS(arg-gly-asp-ser-) and YIGSR (tyr-ileu-gly-asp-ser-) were purchased from Sigma. The YIGSR peptide was also made in our laboratories.

Resin "Fmoc-Arg-PepSyn-KA" and Fmoc-amino acids were obtained from MilliGen/BIOsearch U.K. and dimethylformamide, piperidine,1-Hydroxybenzotriazole, t-amyl alcohol, dichloromethane, diethyl eather,phenol, ethane diethyl, triflouroacetic acid,petroleum ether and diethyl ether were from Aldrich Chemicals.

2.1.8. INHIBITORS

Emetine dihydrochloride, cycloheximide (crystalline) and monensin sodium salt were obtained from Sigma.

2.1.9. RADIOACTIVE COMPOUNDS

^{14}C-Nicotinamide (53mCi/mole, was from Amersham Limited U.K. and [^{35}S]-methionine was purchased from Dupont New England Nuclear).

2.1.10. RADIOACTIVE COUNTING

Scintillation vials were from Packard Instrument LTD. Germany, GF/C discs were from Whatman, Maidstone, Kent, trichloroacetic acid was obtained from Fisons Scientific Apparatus, England. OptiPhase (ethyl substituted benzene) was used as a scintilation liquid and was obtained from LKB.

2.2. METHODS

2.2.1. CELL CULTURE

2.1.1.1 REAGENTS AND BUFFERS.

a) Hepes buffer (20mM):- 12.58 gm of HEPES was dissolved in double distilled water to give final concentration of 20mM and pH was maintained 7.4 with 0.1M NaOH. The buffer was autoclaved (under free steam conditions at 130°C temprature and 15 lb/in^2 pressure for 30 minutes) for sterilization.

b) PBS:- (phosphate buffered saline):- PBS solution was made according to instructions of suppliers i.e. five tablets of PBS were dissolved in 100 mls double distilled water and autoclaved.

c) TRYPSIN:- 1% (w/v) trypsin was prepared by dissolving 10mg of trypsin lyophilisate in 1 ml of pre cooled PBS. 0.1 ml aliquots of this solution were dispensed into sterile storage tubes as quickly as possible, as the trypsin will begin digesting itself and stored at -20°C. Trypsin containing aliquot was thawed immediately before use and diluted in 2ml MEM or EDTA (0.02% w/v) solution.

d) EDTA 0.02% (w/v):- 20 mg EDTA was dissolved in 100 ml of PBS and filtered through a 0.2μm filter for sterilization.

e) CULTURE MEDIUM:- To achieve a 10 fold final dilution of culture medium, MEM and RPMI 1640 (10 x concentration) were diluted in HEPES buffer and D MEM (10 X concentration) in double distilled water.

To these diluted media other constituents were supplemented as indicated below.

2.2.2 MAINTENANCE OF CELL LINES IN CULTURE

All the cell lines tested in the present work were used during the logarithmic phase of growth and maintained in cultures according to the suppliers instructions (Flow, 1989). The old spent culture medium was decanted and monolayers were washed twice with PBS to remove remaining residues of serum.

To detach the cells from the flask, 0.05% trypsin (1% stock solution was diluted in MEM or EDTA (0.02%)solution.) was added and incubated at $37^{o}C$ for 3 to 4 minutes. Whereas MDCK cells required more extensive incubation with trypsin (15 to 20 minutes). At this time trypsin was inactivated with serum containing medium

Cell viability under the conditions used was always typically 99% as was checked by trypan blue exclusion method (equal volumes of cell suspension and trypan blue were mixed and the cells were observed under the microscope). The viable cells excluded trypan blue. The cell lines were maintained as outlined below.

Hela B, CHL, L929 and MDCK cells were maintained in minimum essential medium of Eagle with Earls salt, supplemented with 20 mM HEPES buffer, 10% v/v foetal calf serum, 200 I.U. penicillin, 20 μg streptomycin, 100mM glutamine and 2% non essential amino acids.

BHK21 cells were grown in RPMI 1640 medium supplemented with donor calf serum. 3T3 cells were maintained in DMEM, supplemented with 10% (v/v) foetal calf serum. Walker rat carcinoma cells were maintained in MEM supplemented with horse serum, 0.1% sodium pyruvate. Other constituents for BHK, 3T3 and Walker rat carcinoma were the same as for other cell lines.

Culture medium was always added according to the volumes of flasks (25 cm^2, 75 cm^2 and 150 cm^2) to obtain the cell density of $1x10^5$ cells/ml. Cultures were incubated in a 5% CO_2/air (v/v) atmosphere and were subcultured twice a week.

2.3 MEASUREMENT OF THE CELL ADHESION

For the measurement of cell adhesion the Microflow chamber which was devised in the present work was used throughout this study. This device is shown diagrammatically in figure 3.2& 3.3. The theory, principal and use of this device is illustrated in chapter 3. At this stage it is appropriate to mention that cells are grown on glass or plastic substratum for 24 hours and after this time cell growing substratum is subjected to the hydrodynamic flow in the Microflow chamber for 10 minutes. After this time the Microflow chamber is disassembled and the critical shear stress of detachment was measured by measuring the critical distance and putting its value in a shear stress calculation as described in the chapter 3.

2.4 SERUM AS A STIMULANT FOR CELL ADHESION STRENGTH

2.4.1. GROWTH MEDIUM

2.4.1.1. NUTRIDOMA MEDIUM

Nutridoma medium is a serum free medium, the composition and preparation of this medium for cell culture is outlined as below;

Nutridoma was diluted 100 fold in MEM containing medium. The final medium contained 20mM HEPES, 10% double distilled water, 100 I.U. penicillin, 100ug streptomycin, 100mM glutamine, 2% (w/v) non essential amino acids and 1% Nutridoma medium (Boeringer, 1989).

2.4.1.2. PREPARATION OF MEDIUM WITH DIFFERENT CONCENTRATIONS OF SERUM AND NUTRIDOMA 1%

The MEM medium in separate containers was supplemented with different concentrations of serum, namely 10%,7.5%, 5%, 2.5% 1%, .5% and 0% (v/v). Other constituents of these mediums were the same as illustrated in cell culture section

(2.2), except that these mediums were some times additionally supplemented with 1% Nutridoma medium.

2.4.2 DETACHMENT ASSAY IN RESPONSE TO DIFFERENT CONCENTRATIONS OF SERUM.

Sub confluent monolayers of L929 and Hela B cells were washed briefly with PBS and incubated with 0.01% (w/v) trypsin/.02%(w/v) EDTA at 37°c for 3-4 minutes. After the cells were detached from the culture flask the trypsin was inactivated with 2ml of culture medium containing different concentrations of serum (0% to 10%). The resulting cell suspensions were resuspended in the medium containing 0-10% serum.

In some cultures of L929 and Hela B cells, the trypsin was neutralized by the addition of soybean trypsin inhibitor to a concentration of 0.1% (w/v) in 2ml of Nutridoma medium. The suspension of these cells were inoculated into serum free medium (Nutridoma 1%). Detachment assays were performed with the old and new versions of the Microflow chamber as described in section 2.3.

2.4.3. GROWTH OF L929 CELLS AND MEASUREMENT OF THEIR ADHESION IN SERUM FREE MEDIUM

Nutridoma medium was prepared as revealed above and cryopreserved L929 cells were adapted to this medium over a period of 3 weeks This was done by lowering the concentration of foetal calf serum from 10% to 7.5% to 5% to 2.5% to 1% to 0 %, (v/v) while maintaining the Nutridoma concentration at 1%. The cells were always inoculated at concentration of $2x10^5$ cells/ml and incubated in a humidified atmosphere of 5% (v/v) CO_2/air at 37°C. The health of the cells was continually monitored by checking the pH and whenever necessary replacing old exhausted medium with new Nutridoma containing medium. The cells were very fragile and handled gently. Adhesion strength of finally adapted L929 cells to glass

substratum was measured in term of c.s.s. of detachment under standard conditions outlined in section 2.3.

2.4.4. THE EFFECTS OF SERA OF DIFFERENT ORIGIN ON THE STRENGTH OF DIFFERENT CELL TYPES.

L929, Hela B, and Walker rat carcinoma cells were seeded in the medium supplemented with horse serum or foetal calf serum onto glass or plastic substratum. The c.s.s. of detachment of these cells was measured under standard conditions illustrated in section 2.3.

2.5. THE EFFECT OF FIBRONECTIN AND LAMININ MODIFIED SURFACES ON THE ADHESION OF L929 CELLS.

2.5.1. FIBRONECTIN COATING ON PLASTIC SUBSTRATUM.

Lyophilized human plasma fibronectin was obtained from Flow laboratories. 1mg lyophilisate was dissolved in 1ml of sterilized double distilled water for 30 minutes at room temperature in laminar flow cabinet. The required concentrations of fibronectin were dissolved in 10 ml of sterilized double distilled water. The resulting solution was poured into 100mm tissue culture grade plastic dishes. Fibronectin from this solution was allowed to adsorb on the plastic dishes and the water was evaporated overnight. Dried dishes were washed twice with double distiled water and once with PBS immediately before seeding the cells. Control dishes were prepared in an identical manner except that the first incubation was in 10 ml double distilled water without fibronectin (Obrink. 1982).

2.5.2. QUANTIFICATION OF FIBRONECTIN ADSORPTION.

^{125}I-fibronectin (5.3μCi/μg) in the form of a solution was obtained from Flow laboratories and the same day this solution was made up to 10 ml with double distilled water to give final concentration 1μCi/ml. 0.5 ml of this solution was added per well

of 24 well tissue culture grade dish and allowed to adsorb overnight. The water was evaporated and each well was washed twice with 0.5ml of double distilled water. Both washings were pooled together.

The coated ^{125}I-fibronectin was extracted twice with 0.5ml of 1M NaOH. Each extraction lasted for half an hour. Extractions and washings were counted separately for 2-10 minutes on the gamma counter Curtis & Forrester, 1984).

Together the counts per minute and the known specific activity of the ^{125}I-fibronectin allowed calculation of the number of molecules adsorbed per cm^2, assuming a monomeric uniform distribution. Thus quantity of ^{125}I-fibronectin adsorbed on the plastic was calculated as illustrated below;

Molecular weight of fibronectin = 440 Kd

Or 440,000gm/mole

Or 1µg fibronectn/ $2.3x10^{-12}$mole

Or $2.3x10^{-12} = 12.43x10^6$counts per minute (cpm)

Observed cpm = A

Thus amount in the observed cpm (Y) = $2.3x10^{-12}/12.43x10^6$

Avogadro number = $6x10^{23}$

Number of molecules (B) in Y will be =

$B = 2.3x10^{-12}/12.43x10^6 \times A \times 6.02x10^{23}$

2.5.3 LAMININ COATING ON THE PLASTIC.

100mm tissue culture grade dishes were coated overnight at room temprature with 10 ml of PBS containing 175 µg of laminin. Thereafter unadsorbed laminin was extensively (3-4 times) washed with PBS The control dishes were prepared in exactly the same way except that they were first incubated with PBS without laminin (Shaw et al, 1990).

2.5.4. MEASUREMENT OF CELL ADHESION ON THE PROTEIN MODIFIED SURFACES.

Sub confluent L929 cells were trypsinised, trypsin was inhibited with 10% serum containing medium or with soybean trypsin inhibitor. The resulting cell suspensions were maintained on fibronectin coated or uncoated plastic dishes in the presence or absence of serum in the medium. Where the serum was absent, the medium was supplemented with Nutridoma 1%. The cells were allowed to grow for 3 or 24 hours and after this time c.s.s. of detachment was measured as described earlier (section 2.3).

The adhesion strength of L929 and Hela B cells on laminin coated substratum was examined in exactly the same way

2.6. EFFECT OF RGDS AND YIGSR ON ADHESION STRENGTH OF L929 AND HELA B CELLS

2.6.1. SYNTHESIS OF YIGSR (tyr-ile-gly-ser-arg)

The peptide synthesis was performed on the MilliGen 9050 automated peptide synthesis according to manufacturers instructions (MilliGen/Biosearch, 1990). The MilliGen 9050 pepsynthesizer automates the Fmoc polyamide method of peptide synthesis. The specific amino acid derivatives, support and reagents utilized in this method are described as below.

2.6.1.1. CHEMISTRY OF THE PEPTIDE SYNTHESIS

α) α-AMINO PROTECTION

In the Fmoc-polyamide method of solid phase peptide synthesis, temporary alpha-amino protection is provided by the 9-fluorenylmethyloxycarbonyl (Fmoc) group. The Fmoc group is base labile and can be rapidly removed by beta elimination with secondary amines such as piperidine (structure 2.1).

b) SIDE CHAIN PROTECTION

t-Butyl derived groups are used for side chain protection of L-serine and L-tyrosine (structure 2.2). These acid labile groups are stable during the peptide assembly and are removed at the end of the synthesis by the action of trifluoroacetic acid. Arginine is a special case. The trifluoroacetic acid labile methoxytrimethylbenzenesul-phonyl (Mtr) group is provided for protection of the guanidine function of arginine (structure 2.3)

c) Fmoc AMINO ACID ACTIVE ESTERS

The active esters for the glycine, isoleucine and tyrosine were pentafluorophenyl derivatives (-0Pfp) (structure 2.4) and dihydro-oxobenzotriazine ester derivatives (-ODhbt) for L-serine (structure 2.5).

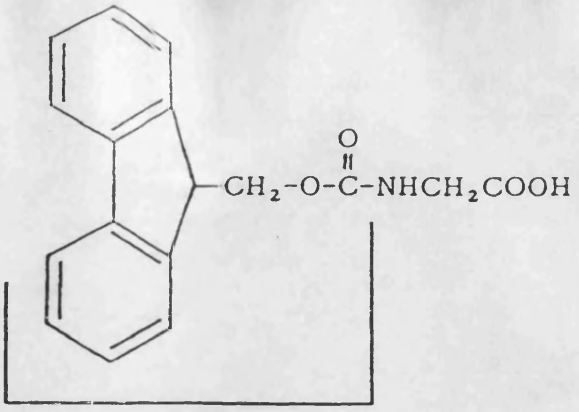
d) COLUMN PACK

d-1) RESIN

In the Fmoc-polyamide solid phase peptide synthesis the support employed was the Fmoc-Arg-pepsyn KA (structure 2.6). This was obtained commercially and according to suppliers information it is formed by copolymerizing a polydimethylacrylamide gel within the pores of rigid macroporous Kieslguhr particles(diatomaceous earth) with cross linking monomers and functinalizing compounds to yield a final resin. The latter, sarcosine methyl ester provides the sites of attachment for the growing peptide. To this Fmoc Arginine is esterified.

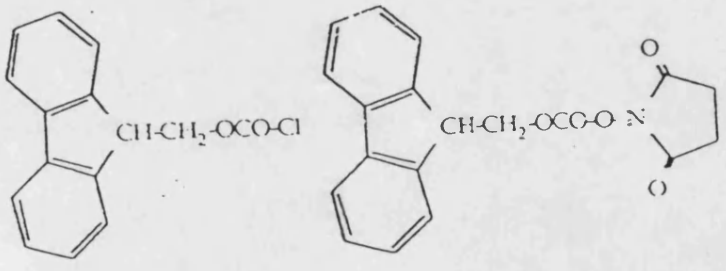
d-2) PACKING OF COLUMN

The dry resin and DMF were gently mixed until the resin was saturated. The column was packed with a fixed length of slurry. An adjustable length end piece was added to eliminate the dead volume and the column was attached to the instrument and proceeded as indicated in section .

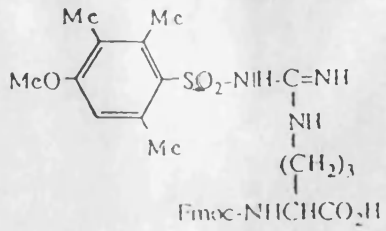


Fmoc Protecting Group

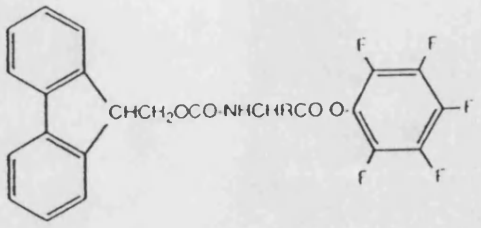
STRUCTURE 2.1 STRUCTURE OF F.Moc -AMINO ACID



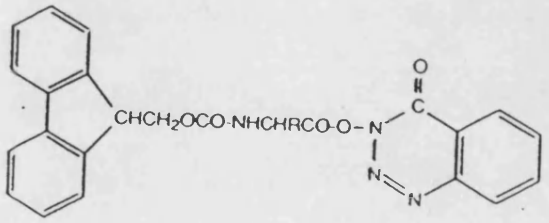
STRUCTURE 2.2 STRUCTURE OF SIDE CHAIN PROTECTOR BUTYL DERIVED GROUPS.



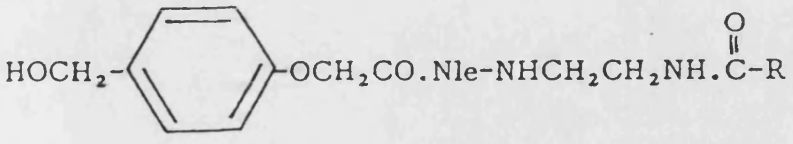
STRUCTURE 2.3 STRUCTURE OF SIDE CHAIN PROTECTOR Mtr GROUP.



STRUCTURE 2.4 STRUCTURE OF ACTIVE EASTERS (-OPfp) FOR AMINO ACIDS



STRUCTURE 2.5 STRUCTURE OF ACTIVE EASTERS (-ODhbt)



STRUCTURE 2.6 STRUCTURE OF PepSyn KA RESIN.

2.6.1.2. REAGENTS AND THEIR USE

a) Dimethylfomamide (DMF)

The general solvent used in YIGSR peptide synthesis was DMF. The solvent contained no amines and was HPLC grade (99.9% pure) (according to suppliers informations).

b) Piperidine

Peperidine was of the highest available purity (99%). It was used as a 20% solution (v/v) in DMF.

c) 1-Hydroxybenzotriazole (HOBt)

HOBt was used as a catalyst to facilitate formation of the ester bond.

d) t-Amyl alcohol

It was used to remove DMF from the column

e) Dichloromethane

It was used as a general wash solvent for removal of t-Amyl alcohol(section).

f) Diethyl ether

Diethyl ether was used to get rid of Dichloromethane and for shrinking of the column.

g) Phenol

It was used for deprotecting (for removal of Mtr) and as a scavenger

h) Ethane diethyl

It reacts with the Mtr and tertiary butyl and thus acts as a scavenger.

i) Trifluoroacetic acid (TFA)

It deprotects the peptide and at the same time it cleaves the peptide from the support.

j) Petroleum ether

It was used to remove TFA but not scavengers and peptide.

k) Diethyl ether

This ether dissolves scavengers and leaves behind precipitated white peptide powder.

2.6.1.3. AUTOMATIC SYNTHESIS OF PEPTIDE

Synthesis was performed on the 9050 automated peptide synthesizer. In this system a MilliGen express- peptide program is provided. This program is a text based software package designed on a nested- menu format. The main menu displays a list of options that may be selected. By selecting different options the job of YIGSR synthesis was assigned to the system. In this regard the following information to the system was provided.

Starting resin = Fmoc-Arg-PepSyn-KA

Resin quantity = 2.2gm

Target peptide length = 5(NH2-Tyr-Ile-Gly-Ser-Arg-COOH)

Molecular weight of the peptide = 594.678

Theoretical yield = 0.118gm

Suggested loop size = 5ml

Average flow rate = 5ml/min

Solution volume for .3 molar = 2.64ml

Estimated reagent usage for synthesis completion:

DMF 524 ml, Piperidine 175 ml and HOBt 11 ml

Chemistry of amino acids	quantit y	position on rack
Gly (Fmoc-Gly-OPfp	0.367gm	2
Ile (Fmoc-L-Ile-OPfp	0.411gm	3
Ser (Fmoc-L-Ser(But)-ODhbt	0.419	1
Tyr (Fmoc-L-Tyr (BUT)-OPfp	0.495	4

The starting resin was soaked in DMF for 10 minutes at room temperature. The resin was washed extensively with DMF to remove fine material. The column was packed as illustrated above and was attached to the instrument. The resin on the column was thoroughly saturated with DMF. The flow rate was 5ml/min and DMF was run for about 2 min. Solvents were further run for 15 minutes to remove air bubbles from the system. The amino acid vials were now loaded in the rack according to the position indicated above. At this point automatic synthesis was started by pressing the key "Run" of computer attached to the MilliGen instrument.

Though the system is automated, it is appropriate to mention that in the Model 9050, a protected Fmoc- amino acid is the starting material. Addition of soluble activating agents (e.g. OPfp & ODHbt) to the amino acids results in the formation of active species. The activated amino acids are transferred to the reaction column for the coupling step. Series of cycles take place. For example, the piperidine, deprotects the amino terminus of a support bound amino acid which in turn reacts with the activated carboxyl terminus of the incoming amino acid. The cycle of deprotection and coupling is repeated until chain elongation is complete.

Completion of the solid phase peptide synthesis results in the generation of a resin bound peptide. The column was removed from the system. The peptide on the resin was first thoroughly rinsed in inert organic solvent i.e. t-amyl alcohol. This was important because DMF was used during synthesis, since it is non volatile and its presence would have interfered with the subsequent cleavage and deprotection procedure. FM1 LAB pump (module G150) was used to wash the column with 40-60

ml of t-amyl alcohol and then to get rid of t-amyl alcohol the column was washed with 20 ml of dichloromethane. Until this stage the column was still swollen and it could still retain some solvents. Thus 20 ml diethyl ether was pumped through the column to wash out the dichloromethane and to shrink the resin gel.

2.6.1.4. CLEAVAGE AND DEPROTECTION OF PEPTIDE

With PepSin KA resin, the peptide resin bond is cleaved and most side chain protecting groups are removed by the action of a TFA/ scavenger mixture. For this purpose to 900 mg of resin bound peptide a pre cooled mixture of TFA and scavengers (0.25g phenol, 250µl ethane diethyl and 10 ml of TFA 95%) was added and reaction was left to proceed for 4 hours at room temperature. Peptide plus TFA/scavenger mixture was filtered through scintered glass and collected in a round bottom flask.The Resin was washed 3-4 times with TFA and the filtrate was collected in the same round bottom flask. To remove TFA and stop the cleavage reaction about 100ml petroleum ether was added to the mixture of cleaved peptide, scavengers and TFA.The peptide was precipitated and filtrate was carefully discarded. To the precipitate diethyl ether was added. Diethyl ether dissolved the scavengers leaving peptide as a precipitate white powder. The precipitated material was dissolved in 0.5% B solution (acetonitrile + water(90:10) + 0.1%TFA).The peptide was dried on the rotary evaporator and freeze dried overnight.

The goal of cleavage/deprotection was to separate the peptide from the support and at the same time to remove the protecting groups from the side chains. This was done quickly to minimize the exposure of the peptide to the acid reagent. The peptide was then recovered from the reaction mixture as stated above and analysed on HPLC.

2.6.2. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

a) BUFFERS

BUFFER A: 0.1% (v/v) aqueous TFA

BUFFER B: acetonitrile in 0.1% (v/v) aqueous trifluoroacetic acid.

2.6.2.1. ANALYTICAL HPLC

HPLC appratus was from LKB and in this a reverse phase VYDAC C-18 column was used for analysis of the collected fractions. The column was 25cm in length and 4.9mm in internal diameter. The calculated flow rate was 0.72ml/min. 0.028 gm of lyophilized peptide was dissolved in buffer B and analytical HPLC was performed by using gradient mode of 5% B and 95% A. The gradient was stopped at 40% B. The peptide was monitored at 215nm which is closer to the absorbence of the peptide bond (210-214). The peptide started to elute at 27% B gradient. About 20 fractions were collected on this gradient and analysed on the preparative HPLC as described bellow.

2.6.2.2. PREPARATIVE HPLC

HPLC apparatus was from LKB in which a C18 column of wide pore(300A^{o}) with a particle size of 10μm in diameter was used. The column was 25cm long and 10 mm in internal diameter. The calculated flow rate through this column was 3ml/min. Preparative HPLC was performed by using the gradient mode of 5%B and 95%A. A large peak at 25% B was obtained with several small "junk" peaks which were just equivalent to the peaks of running reagents. Fractions were collected at this large peak and analysed further.A single peak was obtained at 215nm. Whole cleaved peptide was purified by using the protocol described above. Solvents were evaporated on the rotary evaporator and freeze dried overnight. Lyophilisate was stored at 4^{o}C until use.

2.6.3 EFFECT OF RGDS & YIGSR ON ADHESION STRENGTH OF L929 CELLS

Immediately after plating the cells on plastic or glass substratum for detachment assay, the RGDS or YIGSR (concentrations indicated in the figures 6.1, 6.2 and 6.3) was added. The effect of these peptides on the adhesion strength of L929

cells was analysed by incubating the cells for 24 hours and measuring the c.s.s. of detachment with the old or new version of the Microflow chamber.

2.7. ROLE OF PROTEIN SYNTHESIS IN CELL ADHESION

2.7.1. PREPARATION OF DRUG SOLUTIONS

a) EMETINE OR CYCLOHEXIMIDE (5mg/ml):- 100mg emetine or cycloheximide was dissolved in 20ml complete medium and sterilized by filtering through a 0.2µm filter. 1ml aliquots of this stock solution were dispensed into sterilized Eppendorf tubes and stored at -20^{o} C. The frozen drugs were thawed immediately before use and stock solutions were serially diluted to obtain the concentration of drugs indicated in the figures 8.1 to 8.4 and 8.5,8.8 and 8.9.

b) MONENSIN (5mg/ml):- 100mg monensin was dissolved in 20 ml absolute alcohol and kept in freezer. Immediately before use the monensin solution was warmed at room temperature for 15 to 20 minutes. 100µl of alcohol containing monensin was added into 100ml of Nutridoma or complete medium. From this solution (5µg/ml), serial dilution was performed to achieve a final concentration of monensin as indicated in the figures ,8.5,8.6,8.8, and 8.9.. No adverse effects of alcohol(1µl/ml) without monensin on the growth or viability of L929 cells were observed, as was checked by trypan blue exclusion method.

2.7.2 DETERMINATION OF EFFECTIVE DOSE OF DRUGS FOR INHIBITION OF GROWTH OR PROTEIN SYNTHESIS.

To determine the specific dose of monensin, emetine or cycloheximide to work with, the response of L929 cells to these drugs(in term of their growth or/and protein synthesis) was examined as described below.

2.7.2.1. GROWTH:

Sub confluent cells were trypsinized and trypsin inhibited as stated earlier (section 2.2.2). The resulting cell suspension (1 x 10^5cells/ml) was inoculated with these drugs (concentrations are indicated in the figures (8.1 to 8.4). Cell counting was continued with heamocytometer at different intervals for 96 hours. The measurement of population doubling time was used to quantify the response of L929 cells to these drugs.

2.7.2.2. PROTEIN SYNTHESIS INHIBITION.

For the protein synthesis inhibition following steps have been taken

a) DILUTION OF L-[^{35}S]METHIONINE (8.5mCi/ml):-

L-[^{35}S]methionine was obtained from Dupont. The septum of the vial was pierced with a syringe needle and touching of the frozen product was avoided.The vial was vented in the fume hood and thawed at room temperature. Any pressure developed could vent through the syringe needle. The needle was removed and thrown in the radioactive waste bag. The contents of the vial was diluted with 10 ml of mercaptoethanol (20mM) and aliquotd equally into 10 ependorf tubes and stored at -80^{o} C. Immediately before use this stock solution was diluted in complete medium. The final concentration of radioactivity which was added per well of 24 well plate was always 0.51μCi.

b) DETERMINATION OF PROTEIN SYNTHESIS

L929 cells were placed in a 24 well plate in the presence, or absence, of drugs and the cells were allowed to attach to the wells for 2 hours and metabolically labelled by adding 0.51μci of the [^{35}S]methionine to each well. The incorporation was followed over a period of 8 hours. At times ranging from 0 to 8 hours, the labelled medium was carefully removed and each well was washed twice with PBS. The cells were then dissolved in 0.5ml of 0.1M NaOH which instantly digested the

cells. To this mixture 2 to 3 ml of ice cold 10% TCA was added and the reaction was left to proceed overnight at 4°C. The precipitated samples were then passed through a GF/C disc (previously washed with 2ml ice cold 5% TCA) on an ultrafiltration tower. The discs were then washed with 3 aliquots of ice cold 5% TCA (5ml) and finally with 95% ethanol (2-3ml). The discs were placed in a scintillation vial and dried at 60° C . After drying, 2-3ml scintilant (OptiPhase) was added to each vial. The samples were counted in a Packard Tri- carb liquid scintillation counter.

2.7.3 SECRETION OF PROTEINS

Sub confluent L929 cells were sub cultured into 24 well plates and were metatabolically labelled with [^{35}S]methionine (1μCi/well) overnight in serum containing medium. After this time, the labelled medium was decanted and [^{35}S]-methionine incorporated cells were carefully washed 3 times with Nutridoma medium (0.5ml). To each well 1ml Nutridoma (1%) medium with monensin or without monensin was added. At different time points (as indicated in figure 8.6) the conditioned medium was collected, centrifuged and precipitated with ice cold 20% TCA. The precipitate was processed for counting as illustrated in protein synthesis section.

2.7.4 SECRETION OF NICOTINAMIDE-^{14}C

The Nicotinamide-^{14}C (53mCi/ml) was from Amersham LTD U.K. and was diluted with 50% ethanol to give 25μCi/ml. 20μl of nicotonamide (25μCi/ml) was added to 250ml of complete medium and out of this labelled medium 10ml was added to each flask (25cm^2) at the time of inoculation of L929 cells. The incorporation of labelled nicotinamide in the L929 cells was allowed to occur for 48 hours. At this time labelled medium was carefully removed and the cells were washed with PBS 2-3 times. To the labelled cells 10 ml Nutridoma medium(1%) with and without

monensin was added. The conditioned medium was collected at different time intervals (see figure 8.7). and after adding scintillant (OptiPhase) counted in a Packard Tri-carb liquid scintillation counter.

2.7.5. EFFECT OF ENDOGENOUS PROTEINS ON THE ADHESION STRENGTH OF L929 CELLS

The following approaches have been developed to examine the effect of inhibition of protein synthesis or secretion on adhesion strength of L929 cells:

a):-

The conditioned medium of sub confluent L929 cells growing on glass or plastic substratum was replaced with or without drug containing new medium (serum or without serum). After this cells were further incubated for 3 or 6 hours and c.s.s. of detachment was measured at this each time point (figure 8.5)

b):-

The overnight growing L929 cells were treated with drugs and further incubated for 24 hours. After this time the c.s.s. of detachment was measured as described earlier (section 2.3). The concentrations are indicated in the figure 8.8.

c):-

Sub confluent L929 cells were trypsinized and after inhibiting trypsin with 10% serum containing medium, the cells were subcultured on glass or plastic in presence or absence of drugs (figure 8.9). The c.s.s. of detachment was measured by using the old or new version of the Microflow chamber.

d):-

L929 cells in their logarithmic phase were trypsinised with 0.05% trypsin in 0.02% EDTA and trypsin was inactivated with 0.1% soybean trypsin inhibitor in Nutridoma medium. The cells were plated on glass or plastic in the presence of serum free media with or without drugs. The cells were incubated for 24 hours to measure the c.s.s. of detachment (table 8.1)

CHAPTER 3
THEORETICAL BACKGROUND OF THE MICROFLOW CHAMBER

3.1. TERMINOLOGY

Before illustrating the theoretical background of the Microflow chamber (a cell adhesion measuring device developed in present work) it is appropriate to discuss some terminology involved in the design of this device.

3.1.1. INERTIAL FLOW

In this kind of flow the inertial force (fictitious force) acts on the fluid i.e. a flow in which no external forces are exerted on a fluid is called inertial flow.

3.1.2. REYNOLDS NUMBER

A dimensionless number which is significant in the design of a model of any system in which the effect of viscosity is important in controlling the velocities or the flow pattern of a fluid; equal to the density of a fluid, times its velocity, times a characteristic length, divided by the fluid viscosity.

3.1.3. LOW REYNOLDS NUMBER

When the Reynolds number is much smaller than unity the viscous force dominates over the inertia force so much that the latter plays a negligible role in the flow dynamics i.e. the flow will be smooth.

3.1.4. HIGH REYNOLDS NUMBER

At high Reynolds number viscous force is so small compared with the inertia force that it can be neglected and the flow will not be smooth.

3.1.5. LAMINAR FLOW

Streamline flow of an incompressible, viscous fluid; that is all particles of the fluid move in distinct and separate lines without turbulence. In other words each element of a fluid travels smoothly along a simple well defined path called Poiseuille flow. That is each element starting at the same place (at different times) follows the same path.

3.1.6. TURBULENT FLOW

Motion of fluids in which local velocities and pressures fluctuate irregularly, in a random manner and producing a turbulent boundary layer in which the Reynold stresses are much larger than the viscous stresses.

3.1.7. CONVERGENT CHANNEL

When a change occurs as a decrease in width, relative to the direction of flow, the transition length is referred to as a convergent channel. Flow through a convergent channel is accelerating.

3.2. THEORY OF THE MICROFLOW CHAMBER

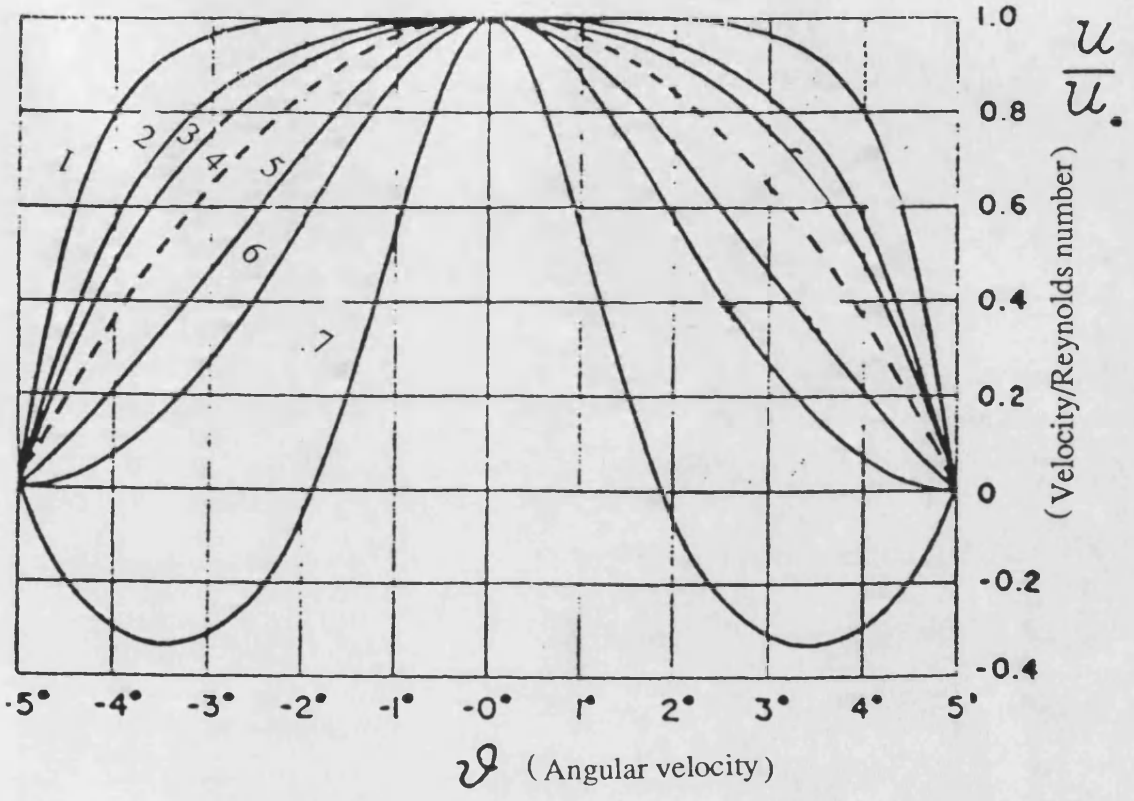
The theory of the Microflow chamber is dependent on the fact that the flow of a viscous incompressible fluid between plane parallel plates is governed by a parabolic velocity distribution (so called Poiseuille flow) (Millsaps & Pohlhausen, 1953). The velocity profiles of fluid in the parallel plate chamber is given in the figure (3.1). An obvious inspection of this velocity profiles show that each of these profiles is more sharply curved in the middle of the channel than the Poiseuille parabola, which is valid for parallel flows.

The velocity profiles also show that as the Reynold numbers increases to still larger values, back flow regions will appear along each wall. In a divergent (widening) channel the profile is not parabolic even at low flow rates and becomes worse as the flow rate is increased until back flow occurs near the walls (curves 5, 6

and 7). However, in the convergent channel, where each of values leads to a symmetrical profile with jets and back flow, the lowest Reynold number or the largest value of K (a parameter which is inverse of the Reynold number) leads to the normal symmetrical profile. In convergent channel, provided the design is correct, the curves 3 to 2 to 1 show that increasing the velocity gives increasingly smooth flow. Moreover it should be noted that Poiseuille flow is smooth parabola (the dotted line of curve 4) and is known as laminar flow. Thus in a convergent channel a wall on both sides of this parabola can be constructed to yield a laminar flow.

The dimensions of the Microflow chamber are given in this section (see later) and are designed on the basis of theoretical predictions that in a convergent channel, turbulent boundary layer becomes laminar, when values of a parameter K (an inverse of Reynold number) exceeds about $2x10^{-6}$, the phenomena which is referred to as "laminarisation". Even if there is turbulence at the entrance to the channel, it becomes laminar very quickly. This is "flow re-laminarisation" and an essential feature of the design of the Microflow chamber. The theoretical studies (Launder & Lockwood, 1969) also show that if the design is correct the values of K increases as the flow is accelerated along the convergent channel. This means flow will be increasingly laminar at higher flow rates.

This laminar flow of a fluid is used as a hydrodynamic shearing force to detach the cells from the surface. It may be argued that cell growing surface is not smooth and the laminarisation of the fluid on the cell growing surface could be turbulent. Again on the basis of the theoretical and experimental evidences (Cebeci & Smith, 1974) it is clear that if the roughness of the surface is reasonably small, nothing unusual will happen to the mean velocity distribution. This is because if the free stream velocity increases rapidly, there is not time for the turbulence to respond very much. In convergent channels, the turbulence fluctuations remain nearly the same in "metere/sec" but the mean velocity in "metre/sec" increases so that the dimensionless turbulence intensity decreases. Thus the flow remains largely laminar.



FOGURE 3.1

VELOCITY DISTRIBUTION IN A CONVERGENT AND A DIVERGENT CHANNEL AFTER MILLSAPS AND POHLHAUSEN (1953)

CONVERGENT CHANNEL	DIVERGENT CHANNEL
Curve 1 = 5,000	Curve 5 = 684
Curve 2 = 1342	Curve 6 = 1342
Curve 3 = 684	Curve 7 =5,000

Curve 4 refers to a channel with parallel walls (Poiseuille parabolic velocity distribution)

This graph shows the velocity profiles of fluids flowing in channels. With parallel walls it is smooth parabola (the dotted line of curve 4) and is known as laminar flow.

and use of the Microflow chamber are given as below.

3.3 INTRODUCTION OF THE MICROFLOW CHAMBER.

The Microflow chamber (a cell adhesion measuring device) is shown, diagrammatically in the figures 3.2 and 3.3. A convergent channel is accurately developed in this chamber, in which a complete laminar flow is achieved for hydrodynamic detachment of the cells from the surfaces. At present two versions of this device exist. The old version is suitable for glass slides and the plastic of the size of the glass slides. The new version is suitable for glass and plastic petri dishes. Both versions are easy to handle and are described below.

3.3.1. OLD VERSION

The Microflow chamber consists of two parallel plates (made by machined perspex) separated by a small distance as illustrated in figure2.1. The convergent channel is designed in the upper part of the chamber. Cell growing plastic or glass slides are inserted into the recess which constituents the lower part of the device. Prior to the channel there is a lead in section (figure 3.2). The two halves of the chamber are assembled and are clamped tight. To ensure the tight sealing a gasket (silicon) is placed around the convergent channel. The Microflow chamber is connected on one side with a reservoir containing running medium and on other side with a peristaltic pump. The device was tapered by (Quatro Biosystem Ltd. U.K.

3.3.2. NEW VERSION

The geometry of this version is exactly the same as the old version. The convergent channel for the new version is tapered in the round shaped aluminium cast and coated with nylon. The cell growing 100mm plastic dish is assembled with this lower part as an upper part of the chamber. The whole assembly is clamped with a metal lid. The inlet and outlet of this version are also connected with a peristaltic pump and reservoir containing medium respectively. (figure 3.3). Actual convergent channel in the new version is

FIGURE 3.2A Three dimensional structure of the Microflow chamber

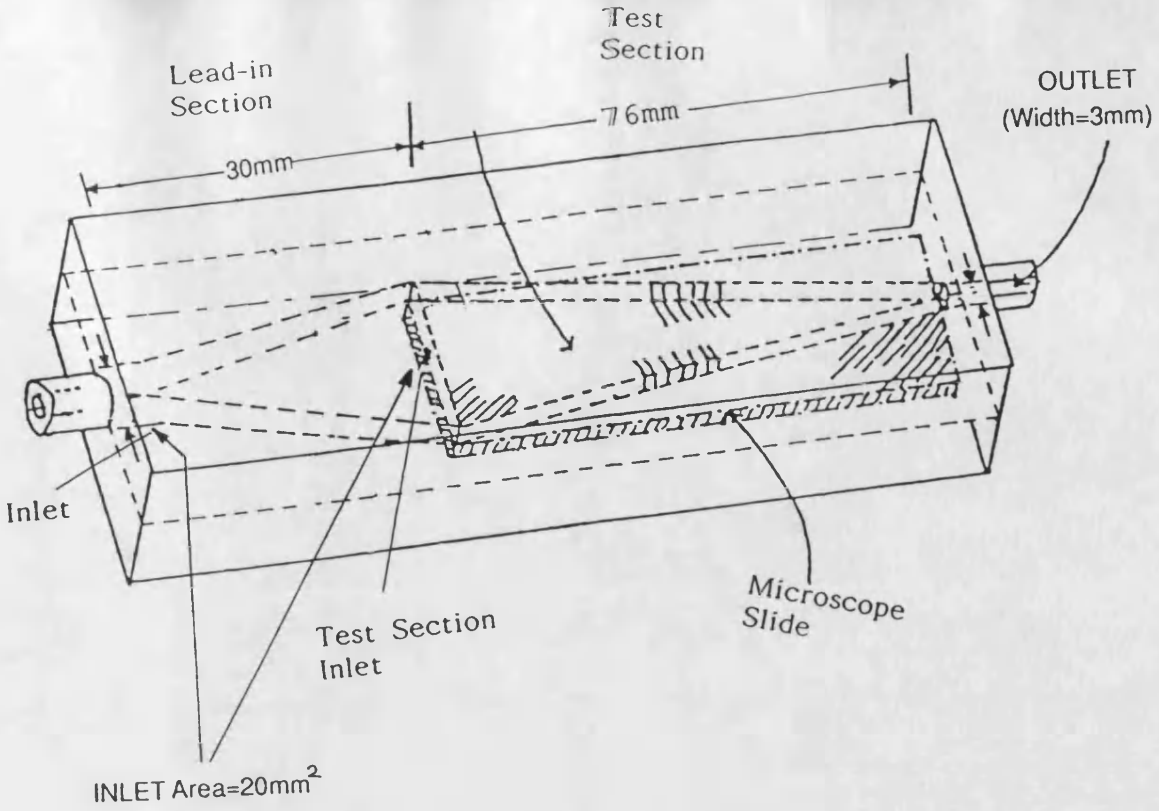


FIGURE 3.2B Cross section of the Microflow chamber

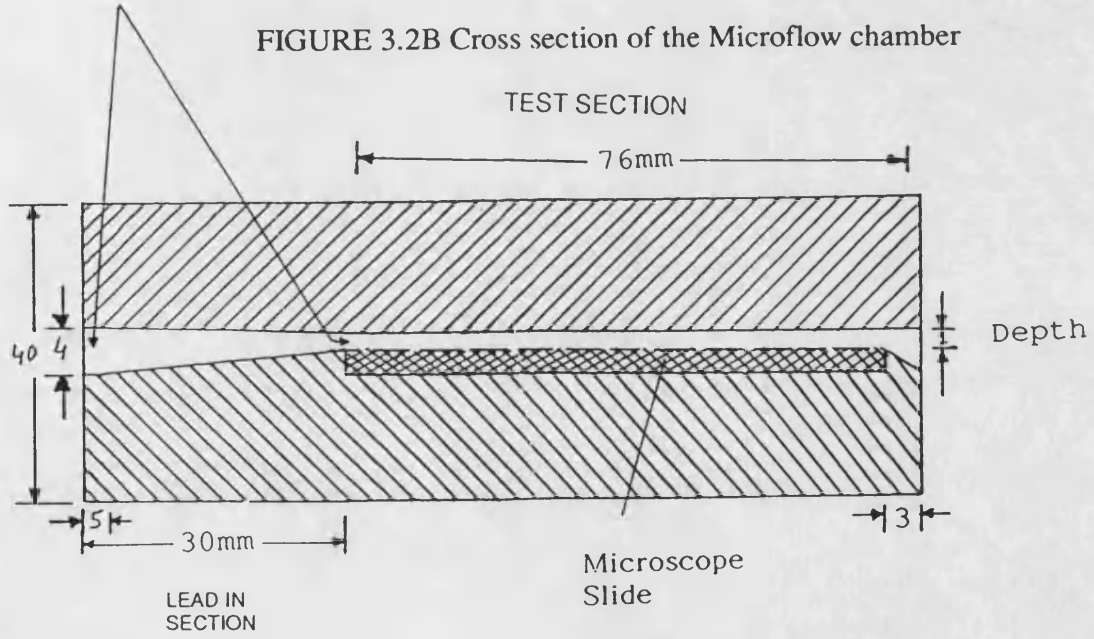


Figure 3.2 Dimensions of the Microflow chamber

FIGURE 3.3 A Dimensions of the Microflow chamber (new version)

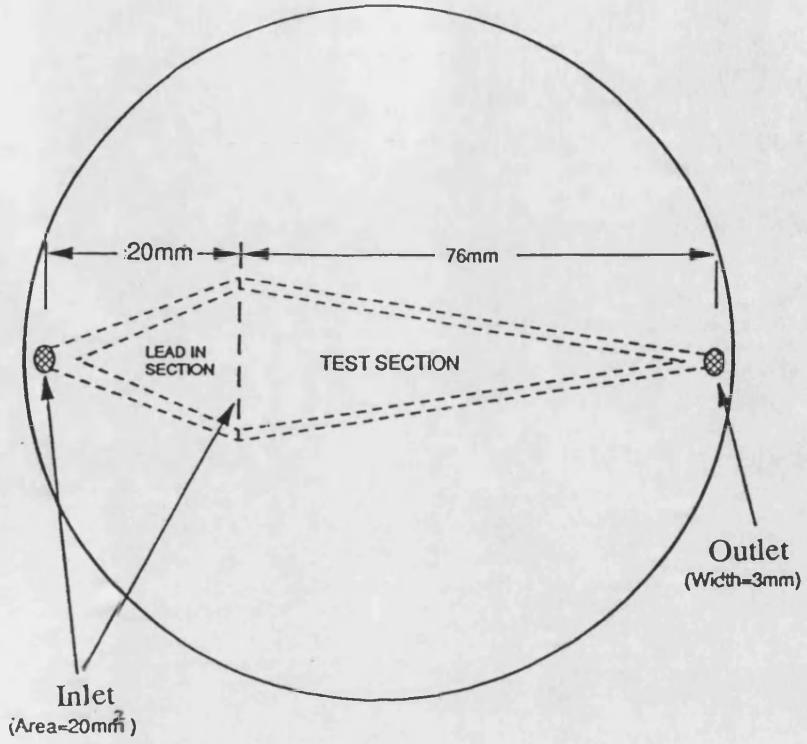


FIGURE 3.3 B Side view of the assembled Microflow chamber

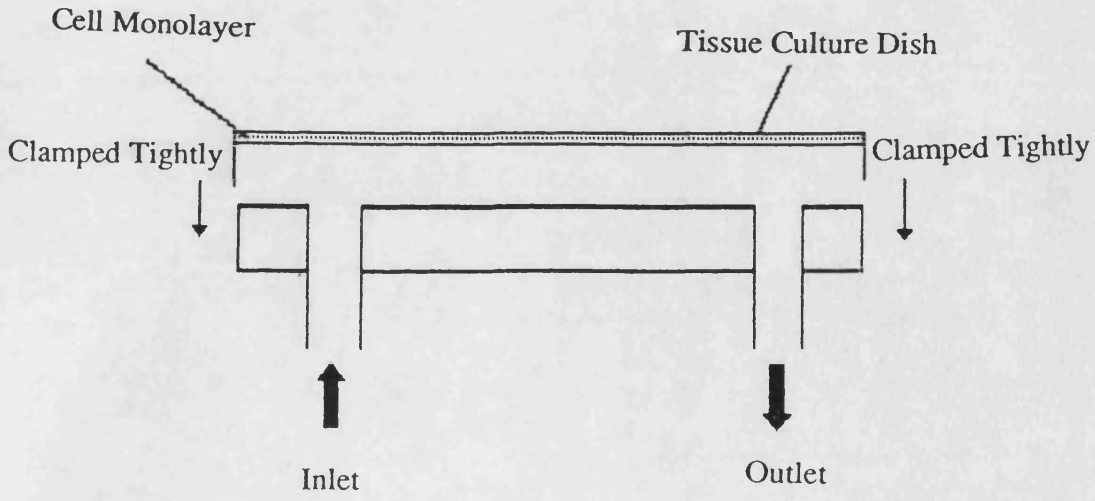
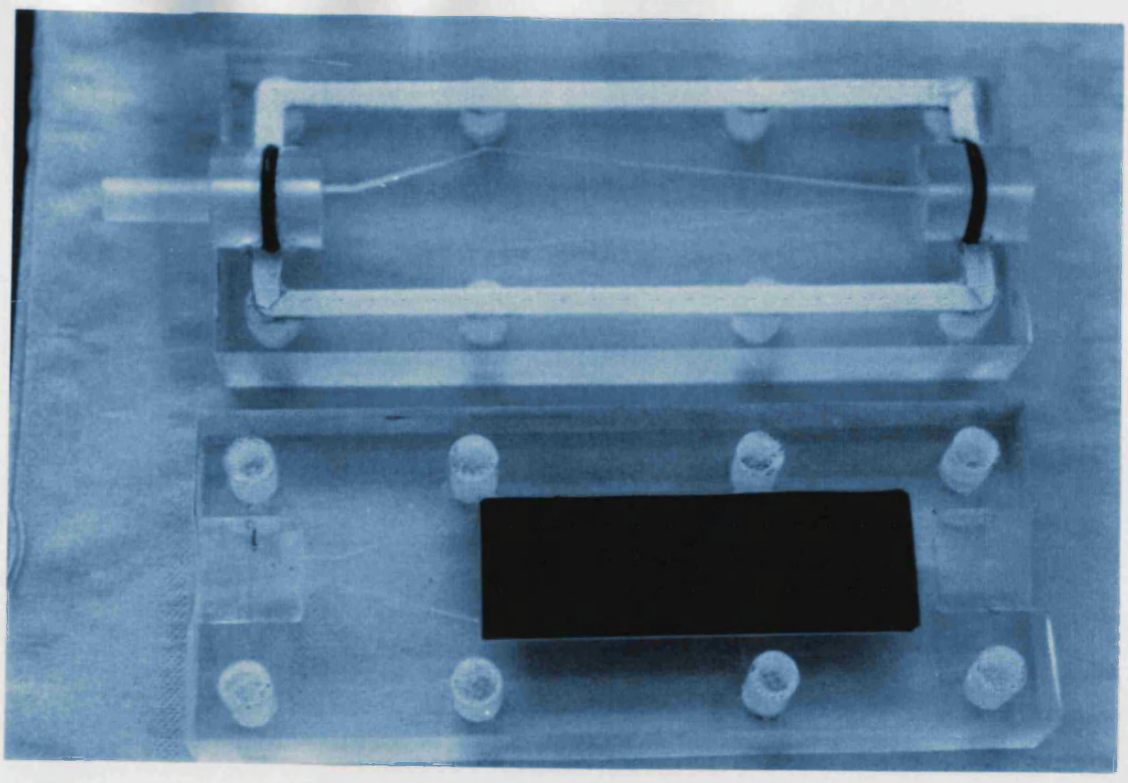
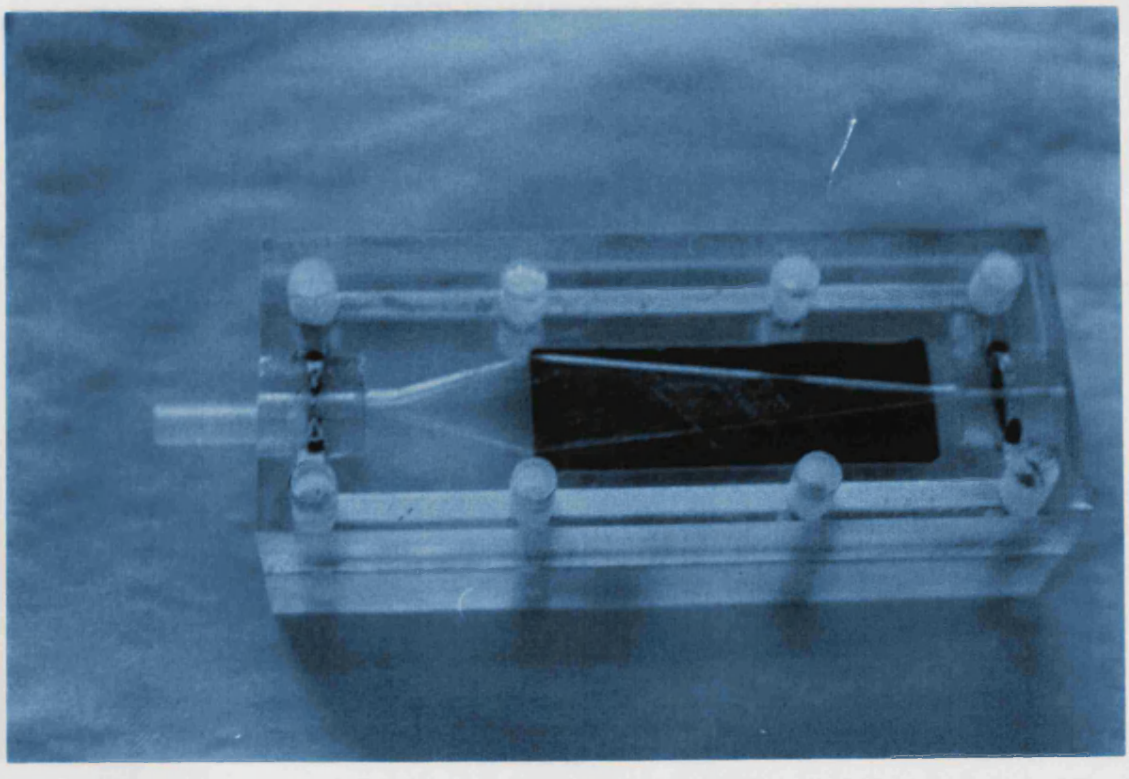


Figure 3.3 The new version of the Microflow chamber

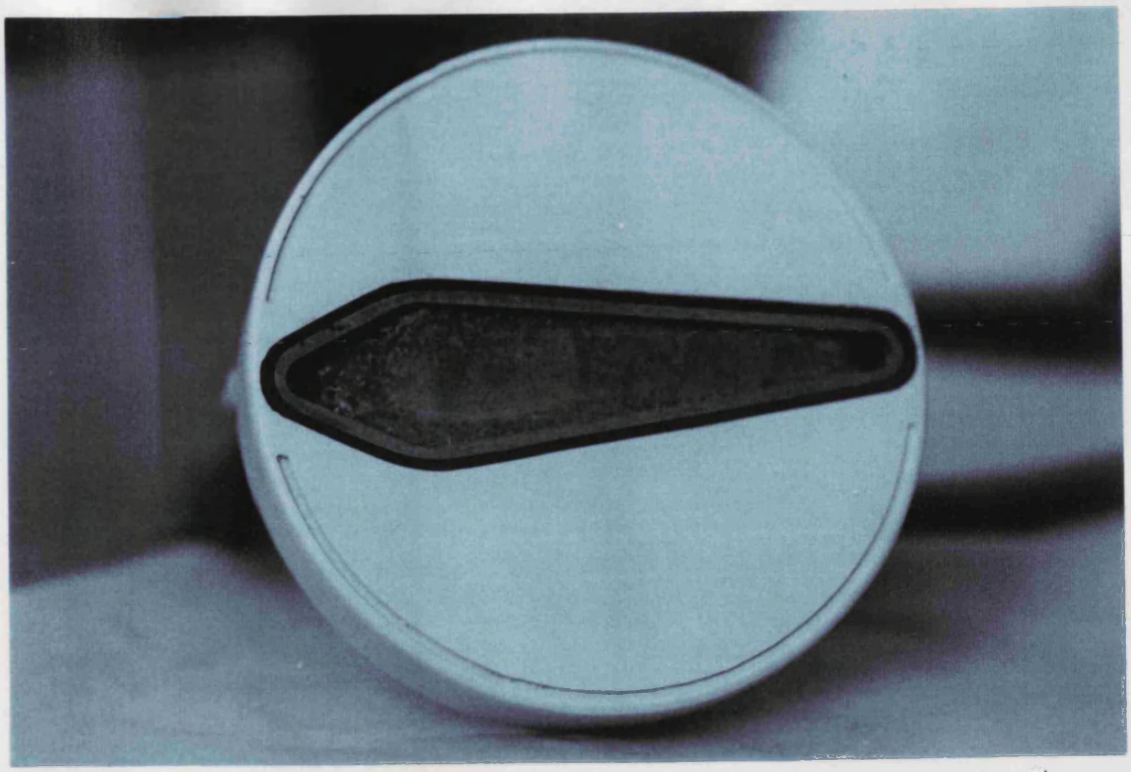


PICTURE 3.1

The Microflow chamber (old version) before assembly



Assembled Microflow chamber



PICTURE 3.2

New version of the Microflow chamber

Cell adhesion assay ot the cells growing in 100mm petri dish.

exactly the same as in the old version. Dimensions of the Microflow chamber are shown in the figure 3.2 & 3.3 and are described below.

3.4 DIMESIONS OF THE MICROFLOW CHAMBER

The old version of the Microflow chamber is 151mm long and consists of two parallel plates. Each plate is 20mm thick (picture 3.1). A convergent channel is devised in the upper part of the Microflow chamber. There are two major sections of the Microflow chamber, a lead in section (30mm) and a convergent channel *i.e* the test section which is 76mm long (figure 3.2). The inlet area of the lead in section is $20mm^2$ and is constant up to the test section inlet which is also $20mm^2$. After the test section inlet, the convergent channel starts, which is tapered by keeping constant depth (1mm). The outlet of the Microflow chamber is 3mm wide.

The new version of the Microflow chamber is 99mm in diameter (picture 3.2).The lead in section in this device is 20mm (figure 3.3).The rest of the dimensions are exactly the same as of the old version.

3.5. DETACHMENT ASSAY

(1) PREPARATIONS OF SAMPLES:-

Sub confluent monolayer cells are trypsinized with 0.05% (v/v) trypsin and the action of this proteolytic enzyme was stopped by serum containing culture medium. The resulting cell suspension, at the concentration of $2x10^5$ cells/ml, was inoculated into complete growth medium. This cell containing medium was poured into $144cm^2$ plastic dishes already containing five sterilized microscope slides or in tissue culture grade plastic dishes (100mm). The cells were allowed to grow for 24 hours in a 5% CO_2/air atmosphere at 37^oc.

(2) RUNNING MEDIUM:-

MEM or RPMI 1640 growth medium was diluted in 20 mM HEPES buffer to give final concentrations of growth medium 10% (v/v). The pH was maintained 7.4 with 0.1M NaOH.

(3) PRINCIPLE AND EXPERIMENTAL PROCEDURE OF THE DETACHMENT ASSAY.

In the old version,the cell growing slide is inserted into the recess which constituents the lower part of the chamber and in the new version,the cell growing dish (100mm) is assembled as the upper part of the chamber. After the assembly of the chamber the running medium from the reservoir is pumped through it at a predetermined flow rate for ten minutes. The flow rate can be controlled by simply varying the RPM of the peristaltic pump (figure 3.4 & 3.5).

As soon as medium enters into the chamber,the lead in section (figure 3.4) reduces the turbulence and stabilizes the flow. As medium enters the test section (figure 3.2 to 3.5) due to the convergent channel, it travels with a tapering width but constant depth, thereby accelerating the flow from the inlet toward the outlet of the chamber. The increasing fluid velocity results in an increase in hydrodynamic shear stress along the cell growing surface (figure 3.6). At a certain critical point, near the outlet, the surface shear stress becomes sufficiently large to cause the detachment of the cells. The critical distance from the inlet to this attachment/detachment boundary is used as a direct measure of critical shear stress (c.s.s.) of detachment in terms of Nm^{-2} (calculation and figure 3.7). The flow rate was calculated by measuring the volume of liquid pumped per minute at a specific speed of the peristaltic pump.

The following equation is derived for the calculation of the critical shear stress of detachment.

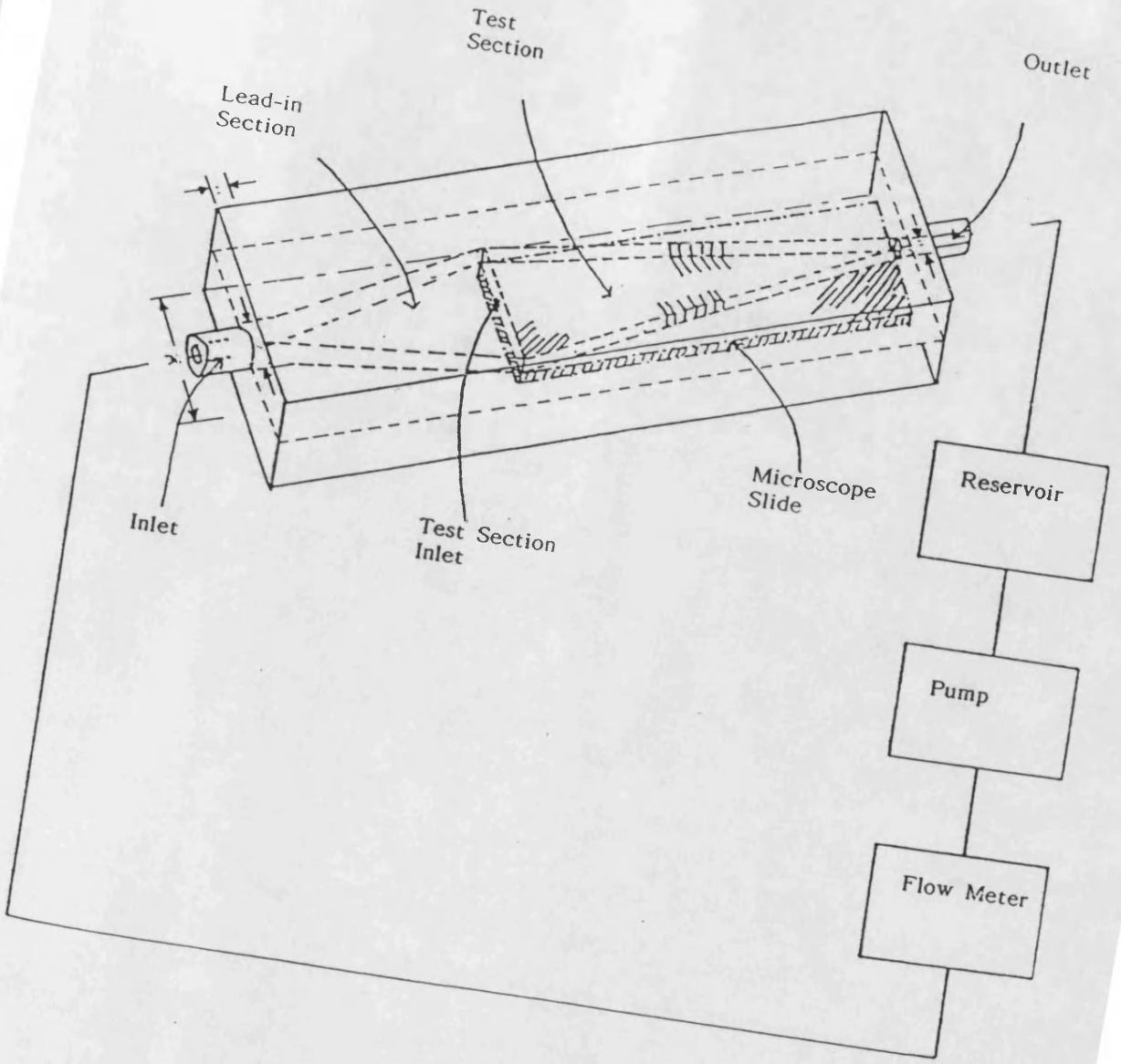


FIGURE 3.4 General arrangements of the Microwflow chamber (old version) and the attached apparatus.

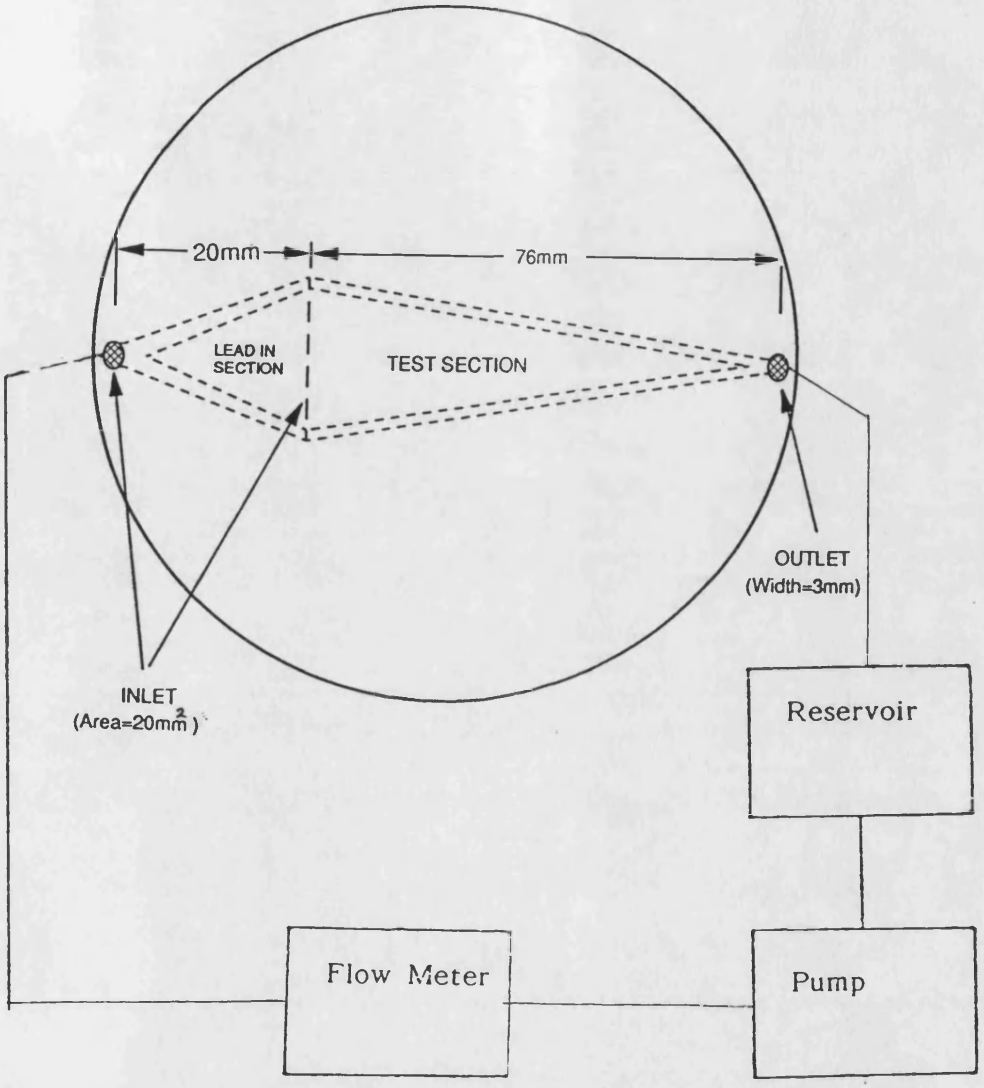


FIGURE 3.5 General arrangements of the Microwflow chamber (new version) and the attached apparatus.

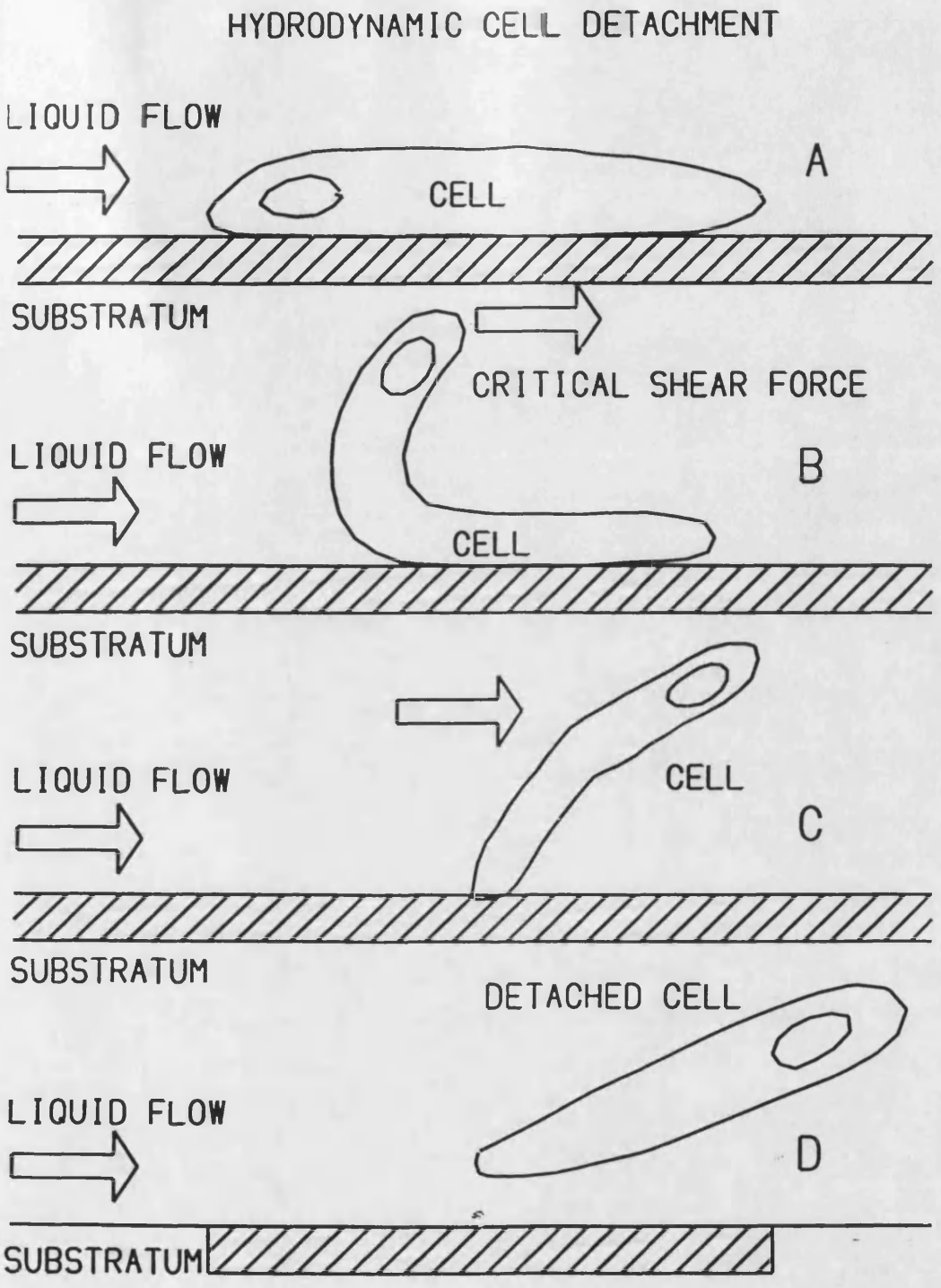


FIGURE 3.6 SCHEMATIC REPRESENTATION OF THE HYDRODYNAMIC CELL DETACHMENT

3.6 CALCULATION OF THE CRITICAL SHEAR STRESS

According to Owens et al(1987) critical shear stress (τ) between two parallel plates can be calculated by using the following equation:

$\tau = \mu \times dv/dy$

μ = viscosity of the fluid

dv = change in the velocity

dy = change in the depth

In the calculation for the critical shear in the Microflow chamber the viscosity and depth are kept constant. Therefore:

Viscosity = μ = 0.012 Poise = 0.0012 Mms^{-2}
Depth = y = 0.5mm = 0.0005m

The third factor in the critical shear calculation is the change in the velocity. By using the dimensions of the Microflow chamber the change in the velocity can be determined as follows:

The fluid enters into the channel through an inlet area ($20mm^2$) with a velocity called the inlet velocity.

Inlet velocity = Volumetric flow rate (f) /inlet area

Flow rate = fml/cm^3s^{-1}

Inlet area = $20mm^2$ = $0.2cm^2$

Thus the inlet velocity = f/0.2 = f/.2 ml $cm^{-1}s^{-1}$

As the fluid travels toward the outlet, the velocity increases in a linear fashion, with an acceleration the ratio of which can be measured as below

Acceleration ratio over the whole test section = Inlet width/outlet width = 20/3 =6.67

The length of the test section is = 7.6cm

The acceleration ratio at any point D will be = 6.67 x D/7.6 = 0.877 x D

There are various streamlines of the fluid which travels with different velocities. The mean of these velocities can be determined.

Mean velocity = Inlet velocity x acceleration ratio at point D ml $cm^{-1}s^{-1}$

Thus by putting the values of these factors in the mean velocity equation:

Mean velocity = f/0.2 x 0.877 x D ml cm^{-1} s^{-1} = f/.2 x 0.877 x D/100 ml m^{-1} s^{-1}

The estimates given by Zachara & Doroszewski (1979) based on the movement of small particles indicate that the velocity of the fluid in the centre will be maximum and 1.5 times more than the mean velocity. Therefore the maximum velocity can be calculated.

Maximum velocity = 1.5 x mean velocity = 1.5 x f/0.2 x 0.877 x D/100 ml $m^{-1}s^{-1}$

Assuming that the velocity has changed from the inlet velocity to the maximum velocity at any point D, the values of the viscosity, depth and maximum velocity (change in the velocity) can be inserted in the equation. for the critical shear stress Nm^{-2}

$\tau = \mu$ x dv/dy = 0.0012 x 1.5 x f/.2 x 0.877 x D/100 /0.0005 Nm^{-2}

τ = D x f x 0.15786 Nm^{-2}

Where point D is the critical distance from the test section inlet to the cell attached/detached boundary and f is the volumetric flow rate for practicle purposes..

The derived factor 0.15786 can be approximated to 0.158.

Thus the final equation can be written as :-

Critical shear stress of detachment (c.s.s.) = D x f x 0.158 Nm^{-2}

SAMPLE CALCULATION

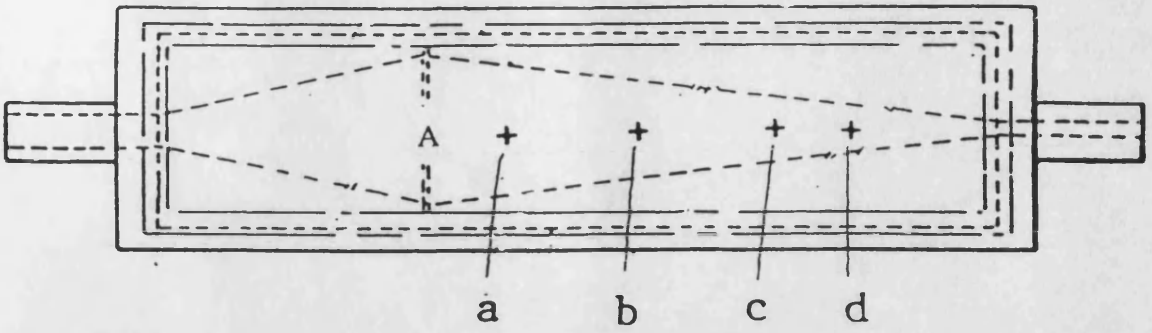
Some examples of the measurement of critical distance and c.s.s of detachment are given in figure 3.6. An example of the calculation of c.s.s. of detachment is given below.

For a critical distance (D) = 60mm = 6cm and a

Flow rate per second (f) = 51 ml

The critical shear stress of detachment (c.s.s.) = critical distance x flow rate x 0.158

= 6 x 51 x 0.158 = 48.3 Nm^{-2}



A = Beginning of the test substratum

+ = Attachment/detachment boundary

Critical distance = (from A to +)

Flow rate = 1000ml/minute = 16.7ml/sec

Critical shear stress Nm^{-2} = flow rate/sec x critical distance x 0.158

Point	Critical distance	Critical shear stress (Nm^{-2})
a	20mm	5.27
b	40mm	10.50
c	60mm	15.80
d	65mm	17.12

FIGURE 3.7

Diagram to show the critical shear stress of detachment at different points (attachment/detachment boundary) along the surface of a cell growing test substratum in the Microflow chamber, at a constant flow rate of 1000ml $minute^{-1}$.

3.7 DISCUSSION

As it mentioned earlier in chapter 1, due to inadequate standard quantitative techniques, the measurement of cell adhesion of different cell lines has been limited and often remained qualitative . Some quantitative studies have been proposed but they all suffer from the need for complex equipment (Horbert et al, 1988; Bowers et al, 1989). Moreover, the lack of reproducibility of previous techniques render them inefficient and incompetent methods (Owens et al, 1987). This necessitated the development of a simple and reproducible quantitative technique for the measurement of cell adhesion.

In the present work these requirements have been met when a simple but reproducible technique for the measurement of cell adhesion has been developed. The heart of this technique is a specially designed Microflow chamber (U.S. patent 4831869).

This Microflow system is the first of its kind in which a complete hydrodynamic laminar flow is achieved through a convergent channel. The convergent channel is designed by keeping a constant depth but tapering the width of the chamber. Prior to the channel, there is a lead in section which stabilizes the flow and reduces turbulence (figure 3.2 & 3.3). The convergent design of the chamber accelerates the liquid flow as it passes from the inlet to the outlet. An increasing hydrodynamic shear stress is generated over the surface of the test substratum (figure 3.2). At a critical distance from the inlet the cells would be detached due to the shear stress they experience. This distance is used to measure critical shear stress of detachment (Nm^{-2}) (see calculation and figure 3.7)).

Since the flow is laminar, the fluid velocity near the walls of the flow channel is assumed to be zero. Estimates given by Zachara and Doroszewski (1979) based on the movement of small particles through a parallel plate chamber, indicate that the velocity of the fluid close to the walls is approximately one fifth of the magnitude of the velocity of the centre. Therefore, the velocity profiles across the chamber is calculated as 1.5 x the average velocity (section---). This assumption is of considerable importance in the estimation of the strength of cell adhesion.

While fluid velocity plays a crucial role in the measurement of cell adhesion the viscosity of this fluid is also an important parameter. In all the experiments described the viscosity was 1.2 centi poise and kept constant throughout the present work (see equation 3.1).

The measurement of cell adhesion while quantitative also needs to be rapid and reproducible. As in the presently designed chamber, the critical distance is measured with a millimetre scale and the resulting critical distance inserted into a simple equation (see calculation in section 3.9.1).

The novel design and principle of the Microflow chamber, make it almost impossible to compare with previously designed hydrodynamic shearing techniques. However it is worthwhile outlining the deficiencies of previously developed methods and explaining the advantages of Microflow chamber for this research work.

Firstly, it is clear that the strength of adhesion of a fully spread mammalian cells (monolayer) to the surface is so large that it exceeds the shear forces generated in previous methods (McClay et al, 1981; Owens et al, 1988). Shiga et al (1985) realized this deficiency in their device and found that red blood cell adhesion on glass and plastic was too great to be measured by their hydrodynamic shearing method.

Considering this situation, any method developed for quantitative measurement of adhesion must produce a range of shear forces that are likely to be encountered in cells. That is; if cells are only weakly attached low shears will be needed whereas for tightly attached cells very high shears will be needed. In the device described here these requirements are fulfilled by having the accelerated flow, described earlier, and a pump which can provide both low and high volumetric flow rates. Thus the final strength of adhesion of any mammalian cell line on any surface can be assessed easily and accurately with the help of this Microflow chamber.

It is important that at very high volumetric flow rates the fluid flow should remain laminar. For example Hela cells have a critical shear of up to 62.0 Nm^{-2} on glass substratum. This figure does not illustrate the erroneous fluid velocity over the cells. For example at 62.0 Nm^{-2} the volumetric flow rate is 3000 ml/minute. At the cell

attachment/detachment boundary the liquid velocity over the cells is 3.9m/sec (see calculation3.9.1). The flow at this velocity is still completely laminar because the theoretical predictions given earlier show that the liquid flow becomes increasingly laminar as the velocity is increases (Millsaps & Pohlhausen, 1953). Thus the adhesion of very tightly bound cells can be determined. Thousands of measurements have been made within a cell line and the standard deviation is remarkably low for a biological system (see chapter 4).

The converging channel avoids any shear forces except those introduced by laminar flow. This permits the measurements of the force necessary to detach the cells from the surface in a reproducible and quantitative manner. It would be fair to say that the present work would not have been carried out with any existing hydrodynamic system. It would not be immodest to suggest that this quantitative system outperforms any thing published to date. Therefore, the Microflow chamber has opened an enormous vista of possible work in cell adhesion. In the following chapters, the reproducibility, accuracy and validity of this chamber will be discussed in detail and the results obtained used to understand the underlying mechanism of cell adhesion and its strength.

CHAPTER 4
COMPARATIVE ADHESION OF DIFFERENT MAMMALIAN CELL LINES ON GLASS AND PLASTIC SUBSTRATUM.

4.1. INTRODUCTION

Despite extensive research concerning the adhesion of different mammalian cells to various types of substratum, the molecular requirements and mechanism by which cells adhere to surfaces has not yet been elucidated. However considerable progress has been made in the identification and characterization of the parameters that are important in the adhesion mechanisms of all cell lines. Such parameters include adhesion proteins, the ability of a surface to adsorb proteins, receptors for adsorbed proteins and finally stabilization and strengthening of cell adhesion by recruiting cytoskeletal elements in and around the adhesion plaques (Burn et al, 1988). Certain evidence suggests that the requirements for adhesion of fibroblast and epithelial cells to surfaces differ in terms of the cell requirement for different extracellular matrix proteins (Terranova et al, 1986). Some studies attributed the differences in adhesion of various cell lines to the ability of a surface to adsorb adhesion proteins. (Grinnell et al, 1977; Knox & Griffiths, 1980).

It should be stressed that these differences in cell adhesion were demonstrated in the attachment and spreading on a qualitative basis. However, the reasons for the differences in adhesion strength of different cell lines remained obscure. Moreover, as far as present knowledge is concerned, quantitative comparison of cell adhesion of different mammalian cell lines has not been possible before this work. As will be revealed in the following discussion, the research described in this thesis shows that cell adhesion is a phenomena which occurs in two phases: the first phase involves an interaction between cell surface receptors and their appropriate ligands which are deposited on the substratum. This requires no metabolic energy from the cell.

In second phase , cells spread and gain attachment strength to their substratum. The first phase has been studied extensively but the second phase is still

very poorly understood. In the present work, an effort has been made to resolve the mysteries of the underlying mechanism of the second phase of cell adhesion. For this purpose the adhesion of various mammalian cell lines on glass and plastic substrata was measured quantitatively with the help of the Microflow chamber (chapter 3). Furthermore, to help understand the underlying mechanism, the basis of subsequent differences in adhesion strength of different cell lines is discussed in the following section.

4.2. RESULTS & DISCUSSION

During the development of Microflow chamber, the reproducibility of the device was analysed, during which work the molecular basis of adhesion strengthening phenomena was studied. For this purpose it was of initial interest to determine whether or not various cultured mammalian cell lines showed similar or different critical shear stress (c.s.s.) of detachment.. The greater the critical shear, the tighter the cells are attached to their substratum. The cells listed in figure 4.1 were grown under the conditions as illustrated in materials and methods. The c.s.s. of detachment of each cell line was measured according to the conditions outlined in materials and methods. It was of interest to note that the cells presented in figure 4.1 show a wide range of critical shear from $2.2 \pm 1.04\ Nm^{-2}$ in Walker rat carcinoma to $62.0 \pm 1.2 Nm^{-2}$ in Hela B cells on a glass substratum. It was encouraging that a high degree of reproducibility within the particular cell line was observed. That is when any cell line removed from liquid nitrogen storage (cryopreserved) and the grown for 72 hours, the measured c.s.s of detachment was always within the value as illustrated in figure (4.4).

At present it is not known why the c.s.s. of detachment is different in different cell lines. Moreover, these values can not be compared as such with other published research work because quantitative data about phenomena of cell detachment by controlled shear force is almost impossible to come by in the literature. However, some theoretical studies have predicted the factors involved in the underlying mech-

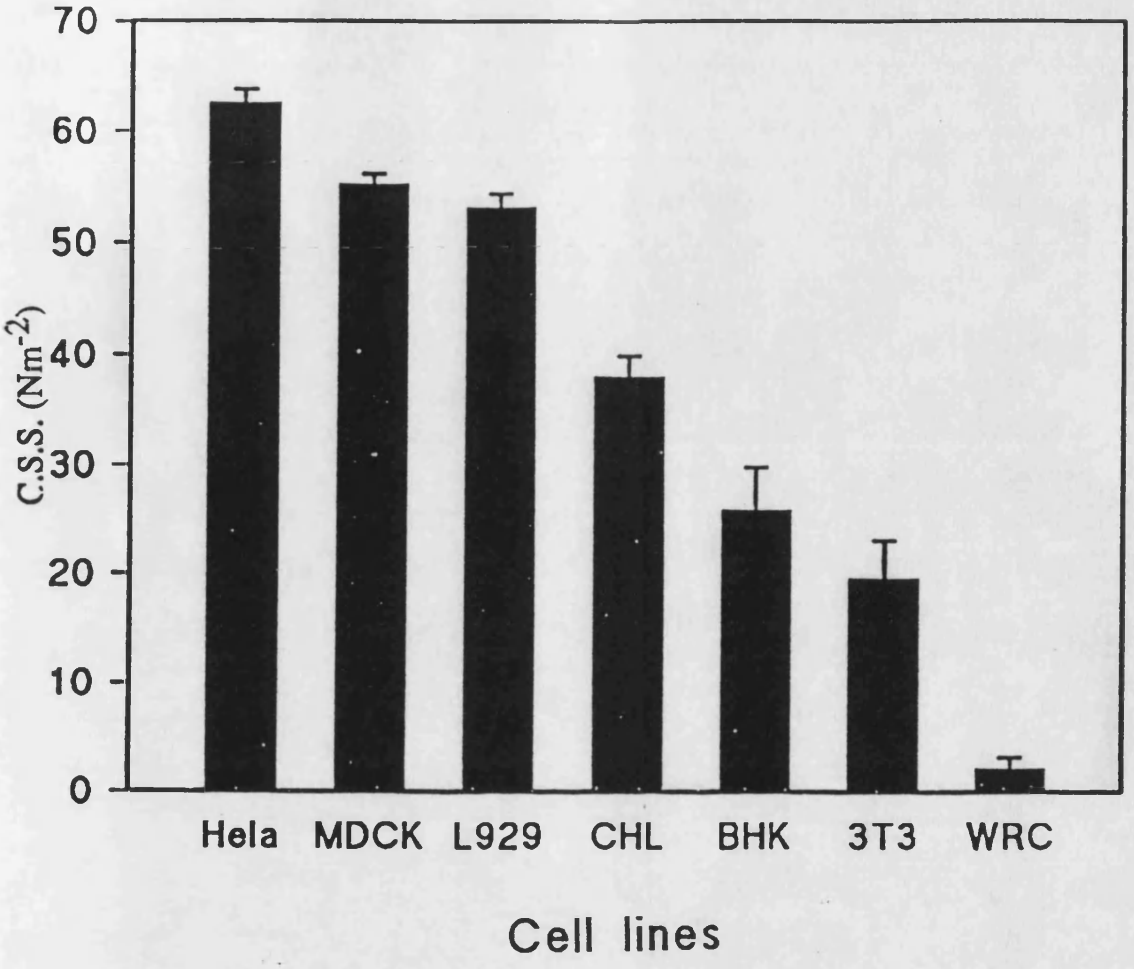


FIGURE 4.1

COMPARATIVE ADHESION STRENGTH OF DIFFERENT CELL LINES ON THE GLASS SUBSTRATUM

Hela B, MDCK, L929, CHL, BHK, 3T3 and Walker rat carcinoma cells (WRC) were grown on glass slides as outlined in materials and methods and adhesion strength of these cells in terms of the critical shear stress (c.s.s.) of detachment was measured by using the Microflow chamber.
Each data point is the mean of 5 experiments each of which contains 20 measurements of c.s.s. of detachment.
The error bars indicate the standard error of the mean. Further details may be found in the text

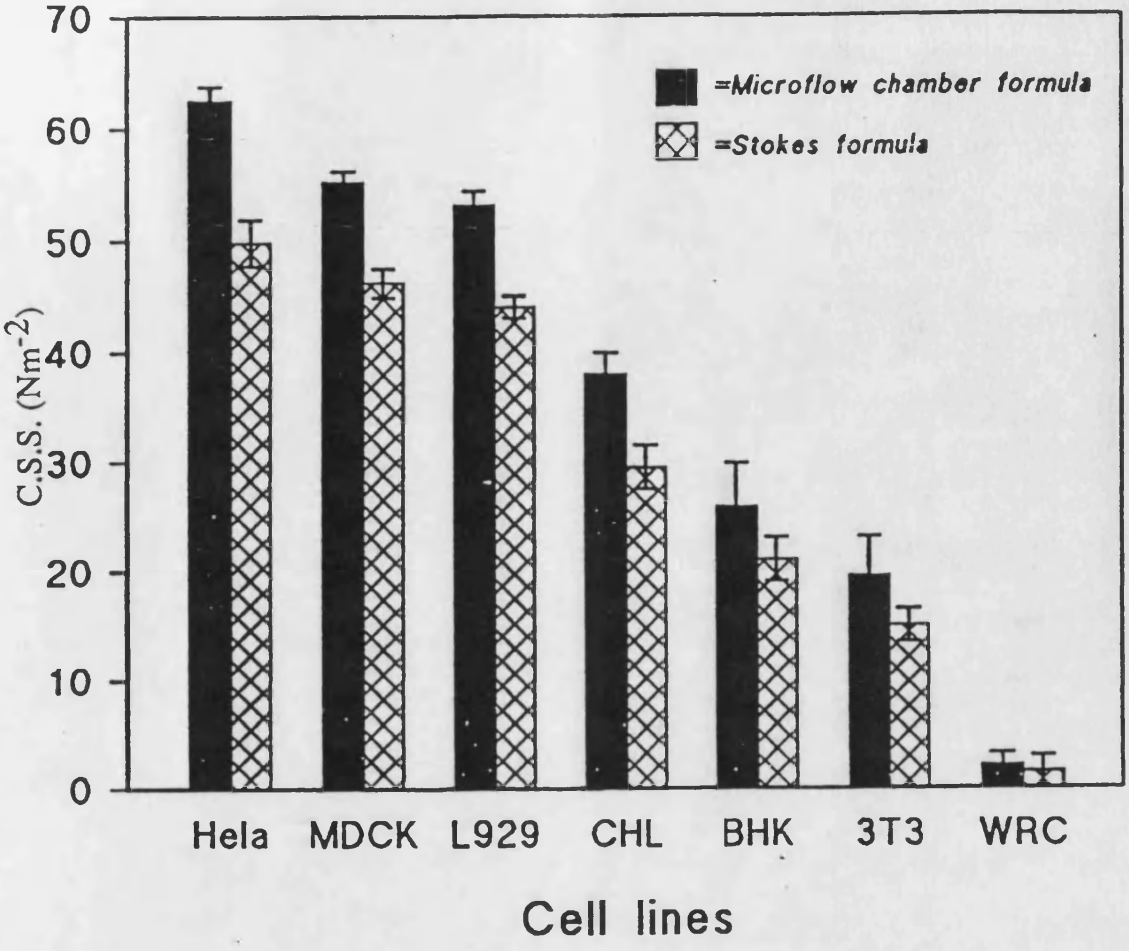


FIGURE 4.2

THE CRITICAL SHEAR STRESS OF DETACHMENT (C.S.S.) OF VARIOUS CELL LINES CALCULATED BY BELLS (1978) THEORETICAL FORMULA LAW AND WITH THE FORMULA DEVELOPED FOR THE MICROFLOW CHAMBER.

Hela, MDCK, L929, BHK, 3T3 and Walker rat carcinoma cells (WRC) were subjected to a defined fluid force (i.e. a known maximum velocity) in the Microflow chamber. The c.s.s. was measured by using the flow rate and the critical distance as illustrated in materials and methods. The c.s.s. of detachment was also measured by inserting the maximum velocity experienced by a cell ($10\mu m^2$) in the theoretical formula given by Bell (1978). Each bar is the mean of 5 independent experiments in each of which 20 calculations were made. The error bars indicate the standard error of that mean. Further details may be found in the text.

-anism of cell detachment (Bell, 1978; Evans, 1985; Cozens-Roberts et al 1990). At present the results presented in figure 4.1 can only be interpreted by speculations and comparing with these theoretical studies. For example Bell (1978) revealed that if a cell of radius r is exposed to a fluid stream of a fixed velocity v the force on the cell can be calculated by Stokes law as described below.

$F= 6\Pi\eta rv$ where η is the fluid viscosity and it is assumed that the flow is laminar. By assuming the area of each cell line tested in this work 10 μm^2 and the maximum velocity they experienced in the Microflow chamber the c.s.s. of detachment of each cell line is calculated. As seen in the figure 4.2, the c.s.s. of detachment calculated according to Stokes law is remarkably close to the critical shear values determined by the formula developed for the Microflow chamber in present work. An acknowledged weakness of this comparison is that the area of every cell line could not be detected in the present work. However, every cell line detached at a specific velocity and this helped to calculate the c.s.s. of detachment (by keeping the area of each cell line $10\mu m^2$).

Although the values calculated by Stokes law are slightly lower than the values calculated by the formula for the Microflow chamber, it is of great interest that entirely different formula of specific design (Microflow chamber) gives similar results to that of theoretical predictions of Bell (1978) about Stokes law. Since both types of calculations proved that every cell line tested with Microflow chamber has its specific critical shear value, the possible reasons for this specificity are given as below.

According to theoretical studies (cited above) , the adhesion force between cell and a surface is the result of the net contribution of the biochemically specific forces. Almost all of these studies are agreed that non specific forces (Van der waals, electrostatic repulsion and steric stabilization) play a negligible role in the adhesion strength of a cell line.

The biochemical forces are believed to largely arise from the level of surface complexes formed from receptors/ligands. These include extracellular ligands

supplied in the serum which are also produced and deposited by the cells themselves, receptor numbers, cell to substrate contact area and increased cell spreading. These parameters are overlapping and crucially interdependent. For the sake of simplicity and ease the role of individual factors in present study will be considered separately.

4.2.1. LIGAND-RECEPTOR BONDS

Theoretically it has been known for some time that the number of bonds between adhesion proteins and their receptors are involved in the adhesion phenomena (Bell, 1978). An unsophisticated but working hypothesis is that the adhesion of a cell for its substratum will increase as the number of receptors increase. This will lead to an increased resistance to hydrodynamic shear. This seems to be the case with the cells bearing higher critical shear values. If it is true, exactly opposite phenomena may be operative in the case of cells which detach at very low critical shear value (e.g. Walker rat carcinoma in figure 4.1). The interactions between adhesion proteins and their receptors may be affected by various factors such as receptor numbers, the density of ligands (adhesion proteins), the affinity of receptors to such ligands and provision of the suitable surface for the formation of bonds.

Apart from these considerations the molecular conformations of the entities taking part in the attachment process are also expected to affect the apparent adhesiveness of the cells. Therefore, variations in c.s.s. of detachment of cell lines listed in figure 4.1 could be attributed to various quantities and qualities of receptors and their ligands (adhesion proteins). For example the variation in c.s.s. might be predicted to be due to interactions with different strengths because the various proteins involved in the interaction may recruit different class of receptors (Dejana et al, 1988). That is the strength of binding of different adhesion receptors e.g. vitronectin inegrin or fibronectin integrin will vary in the same way as the binding strengths of immuonoglobulins to their antigens. In fact the complex multipoint binding between antibody and its antigens might be a good model, at least as an aid to visualise the mechanisms of binding of adhesion proteins and their receptors i.e. some

bind very tightly and some only weakly. Thus the total adhesion strength of a cell for a surface will be the sum of a very wide range of receptors- proteins complexes.

An added complexity is that, for example, vitronectin receptors from different cell types differ in their affinity for vitronectin, yet no chemical difference was found between such functionally different receptors (Languino et al, 1988). It is likely that differences in glycosylation levels of the receptors could explain the above.(Kitagaki-Ogawa et al, 1990). However the spreading of BHK cells on vitronectin isolated from different animal sera was indistinguishable from that on human vitronectin. This point will be further discussed in chapter 5. At present it is appropriate to mention that although vitronectins from different sources equally induced spreading in BHK cells, it could well be that the strength of adhesion of BHK cells varied according to the source of vitronectin. It has not been possible to measure such differences until the Microflow chamber was developed, these differences might now be recognised.

Taken together these observations and results presented in figurem 4.1 it is a general impression that a particular cell adhesion protein may be mediating adhesion strength within the particular cell line, but with different proteins for different cell lines. It is possible that some other proteins also make an important contribution to the adhesion strength by cooperating with this main adhesion protein. It is convenient to call the former the principal adhesion proteins and the latter reinforcing proteins.

There are two possibilities: first, the principal adhesion protein is different in different cell lines or is expressed differently, that is either qualitatively or quantitatively. The second possibility is that reinforcing proteins are different in different cell lines. They may also be expressed in various amounts or in different conformations. In both of these situations bonds of different strengths will exist and thereby, assign a specific adhesion strength to an individual cell line.

One of the simplest systems is the receptor-adhesion protein bonds. Bell(1978) suggested that if multiple bonds underneath the cells had to be detached as a unit, there would be a critical force per bond at which the cells will suddenly detach. This force for a typical antigen-antibody bond was estimated as $1x10^{-5}$ dynes per bond.

Therefore the total force for the detachment of a cell would be $1x10^{-5}$ times the number of bonds in the contact area. It is not possible to compare this force with the force which is applied to the cells in the Microflow chamber. The force provided in the Microflow chamber has a vector while Bell (1978) estimated perpendicular force for breakage of bonds. However,it seems clear that there must be a relationship between the critical shear stress of detachment of a cell and the number of bonds in the contact area. The data presented in this thesis (chapter 8) shows that this might be an oversimplified model. That is the actual measured critical shear of detachment can not only be ascribed to the number of bonds. There must be other factors involved in the final adhesion strength of cells. Thus the theoretical suggestions made by Bell and those published recently (Wattenberger et al. 1990) may not be representing the active process of cell adhesion. It may be that initial binding of adhesion proteins and receptors is only causing the first phase of adhesion which leads to the second phase and collectively both of these phases are responsible for the adhesion strength of a cell. Therefore, there can be little doubt that receptors and ligands are actively engaged in cell adhesion, but the final strength of cell adhesion can not be ascribed solely to ligand- receptor binding.

As was indicated earlier, cell adhesion can be thought of as a two step process. The first step is an initial binding, followed by a substantial strengthening of that binding. The initial binding may represents the attachment of cells to the substrate components under conditions where cytoskeletal events are prevented from participation. In the present work the final strengthened adhesion measured after growing cells for 24 hours represents the initial adhesion plus what seems to be the consequences of coupling of the adhesion receptors to the cytoskeleton. This idea gains support from experiments cited in chapter 7, whereby there is considerable difference between adhesion strength gained by the cells after 3 and 24 hours of growth.

4.2.2. POSSIBLE INVOLVEMENT OF THE CYTOSKELETON

From the data described here and in the chapter 7 the following theory could be developed to understand the underlying mechanism of the final adhesion strength of a cell.

Normally functional cells are able to recognise and measure trace concentrations of adhesion proteins (ligand-receptor), then store and process the information obtained from ligand- receptor bonds to make a decision on further adhesion on the basis of this information. In other words, after binding with the adhesion protein(s) the intracellular portion of the receptor could also bind with the cytoskeleton and thus convey positional information to the cell machinery. This thought is strengthened by the findings that the cytoskeletal protein, actin, provides the structural support to the cell (Burridge et al, 1988). Now, vinculin interacts both with talin and α-actinin which in turn has an additional affinity for actin fibres. In fact a series of protein- protein interactions can be mapped out extending from the extracellular matrix through adhesion plaques to the actin fibres. Although not certain, these events could increase the strength of cell adhesion. Since the actin fibres provide structural support for the cell and in response to shear stress these fibres were found reorganising themselves, actin is thought to participate in the stabilization of adhesion of a cell.(Wechezak et al, 1989). This process may be varied from cell to cell in present study. Thus resulting in the variation of c.s.s. values among the cells shown in the figure 4.1. If the involvement of cytoskeletal events are taken into account, the force required to break the adhesion bonds would be far greater than predicted in the theoretical studies discussed earlier (Bell, 1978).

4.2.3. PARTICIPATION OF THE ADHESION PLAQUES

Adhesion plaques are sites where cytoskeletal events take place. Although focal adhesion is not essential for cell attachment and spreading, its presence is correlated with increased strength of cell adhesion (Burridge et al, 1988). In these regions the surface of the cell comes closest to the substratum, the plasma membrane

is specialized for anchoring stress fibres and microfilaments. Therefore the adhesion plaques might be functioning by preventing the uplifting of the upstream cellular edge , thus, minimizing the hydrodynamic shear traction forces in the Microflow chamber. It is possible that the cells with higher shear values possess adhesion plaques in which stress and microfilament fibres are highly organised. In these cells the distance between substratum and cell membrane at adhesion plaques is expected to be lower compared to the distance of the cells with lower shear values. How cells regulate the information of adhesion plaques and coupling of the cytoskeletal proteins to the protein- receptor complex in and around the adhesion plaques is simply not known. It is unlikely that this type of binding between cytoskeleton and protein-receptor complex is simple "glue" type binding, rather it is likely that there must be something complex going on in the cytoplasm which regulates all the events involved in strengthening the cell adhesion.

4.2.4. SIGNALLING MECHANISM

My impression is (as seen from the fibronectin experiments in chapter 7) that receptors first undergo membrane clustering and then concentrating in focal contacts in a ligand controlled way. Thus the signal is generated by adhesion protein(s)–receptor(s) complex and unknown second messenger(s) receive it and control the later events in a sophisticated manner. The next point to make is to question which molecule acts as a second messenger. At present this is not known but cAMP and phosphatidyl inositol can be included in the list of suspected messenger molecules. There are some indications for the participation of cAMP in the strengthening of cell adhesion (Pastan & Willingham,1978 ; Cheung & Juliano. 1985). That is the treatment of fibroblast cells with cAMP can alter morphology with apparently adhesive cells. It could be that a ligand-receptor complex on the cell surface triggers a short lived increase in the intracellular cAMP In turn the cAMP , activates a cAMP dependent protein kinase system. Protein kinases are thought to be involved in stabilizing the receptor– cytoskeletal coupling (Issaad et al, 1989). Although little is

known about the other functions of cAMP and protein kinases in cell adhesion system, many lines of evidence point to a role for protein kinases in cell adhesion. Phosphorylation is an attractive hypothesis where cAMP dependent kinases regulate the affinity of integrin receptors for ligands or cytoskeletal components (Freed et al, 1989). On one hand phosphorylation is expected to increase the affinity of receptors for the cytoskeleton and stimulate the cells to gain the greater adhesion (Suzuki et al, 1987) on other hand phosphorylation of the fibronectin receptor was shown to induce disruption of cellular adhesion and actin orgnisation (Hirst, et al, 1986). Taken together, these observations suggest that both positive and negative controls are operative. It is possible that the cells use both these controls in concert to allow itself to grow and migrate. Nevertheless it seems apparent that adhesion is perturbed or manipulated by the cell using various mechanisms.

There are other factors which may increase or decrease the adhesion strength of cells. Some of these are listed below.

4.2.5. PROTEASES

An additional cause of variability in the cells responses to critical shear may be attributed to the degree of activation of cell surface proteases in an individual cell line. The proteases may be involved in the degradation of extracellular matrix components and/or cytoskeletal proteins (Fox et al, 1985) Thus resulting in the disruption of binding of integrin receptors to the extracellular cytoskeletal proteins. In fact a specific example is the cleavage of talin or vinculin by Ca^{2+} dependent proteases which represents a potential mechanism for regulating adhesion plaque organisation and thus the subsequent strength of cell adhesion (Beckerele et al, 1987). Again proteases are themselves believed to be activated by phosphorylation. It is possible that adhesion proteins send a signal to the cell machinery to activate these proteases by a greater amount in those cells which are only loosely adhered (e.g. Walker rat carcinoma(2.2 ± 1.04 Nm^{-2})., conversely a lower degree of proteases

activation will occur which are tightly attached to the substratum (e.g. Hela B 62.0 Nm^{-2}).

4.2.6. ANTIADHESION PROTEINS

In contrast to the adhesion proteins there are some secreted glycoproteins e.g. tenascin and SPARC (secreted protein acidic and rich in cystine) which exert an antiadhesive effect on the cells (Villarreal et al, 1989; Faissner and Kruse et al, 1990). The mechanism of their anti adhesive action is yet to be determined. There is considerable speculation as to how these antiadhesive molecules work. For example, antiadhesive function was attributed to an interference with focal adhesion (Mosher,1990) or modulation of protease activity (Hasselaar et al, 1991). Several investigators have in fact presented a strong case for functional antagonism between fibronectin and tenascin (Chiquet-Ehrismann et al, 1988). In my study, the relative amount of each of these proteins may determine the specific adhesion strength to each cell line. These studies suggest that the cells with lower critical shear value (poorly adhesive) might be expressing tenascin or tenascin like molecules in relatively high amounts. For example the antiadhesive molecules might be acting as a blocker or inhibitor of binding of cellular receptors with other extracellular components, thus interfering in receptor-ligand bond formation. This event perhaps blocks the signal generated by adhesion proteins on the cell surface. This might explain the weak cell adhesion strength of Walker rat carcinoma cells. On the other hand it might be that L929, Hela B and MDCK cells are negative in tenascin and therefore showed high critical shear value. The role of antiadhesive molecules and their various mechanisms is yet to be determined. If it is true, tenascin or tenascin like molecules are probably working under the control of cellular negative signalling.

4.2.7. DEGREE OF SPREADING AND ADHESION STRENGTH

There is reason to believe that the critical shear force is not entirely dependent on the degree of spreading. The morphological studies carried out in the present work

have substantiated this idea. For example, MDCK cells are more spread (have a large surface area) than the Hela B cells on a glass substratum yet their c.s.s. value (55.2 $\pm 0.84\ Nm^{-2}$) is lower than that of Hela B cells ($62.0 \pm 1.2\ Nm^{-2}$). This idea also gains support from the findings that the flattened morphology of a adhesion defective 3T3 mutant could be restored by the treatment of cAMP and yet their adhesiveness to the substratum decreased (Pouyssegur & Pastan, 1976). Taken together these findings and the morphological studies in the present work suggest that adhesion strength may not be solely determined by the spread area of the cell. However, one must not imply that spreading is irrelevant to the adhesion strength.

The role of the substratum in cell adhesion has been studied in cell adhesion for many years since the poineering work of Curtis (1960). It is clear that a cell adhesion to a surface depends on the wettability of that surface. In other words its hydrophobic and hydrophilic nature (Grinnell, 1978; Hattori et al, 1985). However there has never been a quantitative study relating adhesion strength of a cell to the properties of the surface. With this in mind a study was carried out to examine the c.s.s.. of detachment of different cells on different surfaces. For this work, tissue culture grade plastic was selected and adhesion strength of different cell lines was analysed on this surface.

4.3. COMPARATIVE CELL ADHESION ON PLASTIC SUBSTRATUM.

The cell lines shown in the figure 4.3 were subcultured in round tissue culture grade plastic dishes (100mm) (see materials & methods) and c.s.s. of detachment was measured with a new version of the Microflow chamber as described in materials and methods. It was exiting to find that like glass a wide range of c.s.s. of detachment was found, which varied from $10.2 \pm 1.5 Nm^{-2}$ in Walker rat carcinoma to $70.5 \pm 1.9\ Nm^{-2}$ in MDCK cells (figure 4.3). However, it is clear that all the cell lines tested with the exception of CHL cells, stuck better to plastic than glass. That is, the c.s.s. values ranged from 9.5% increase in glass c.s.s. values for Hela B to 70% increase in glass c.s.s. values for Walker rat carcinoma cells. (figure 4.1 & figure 4.3). As can be seen

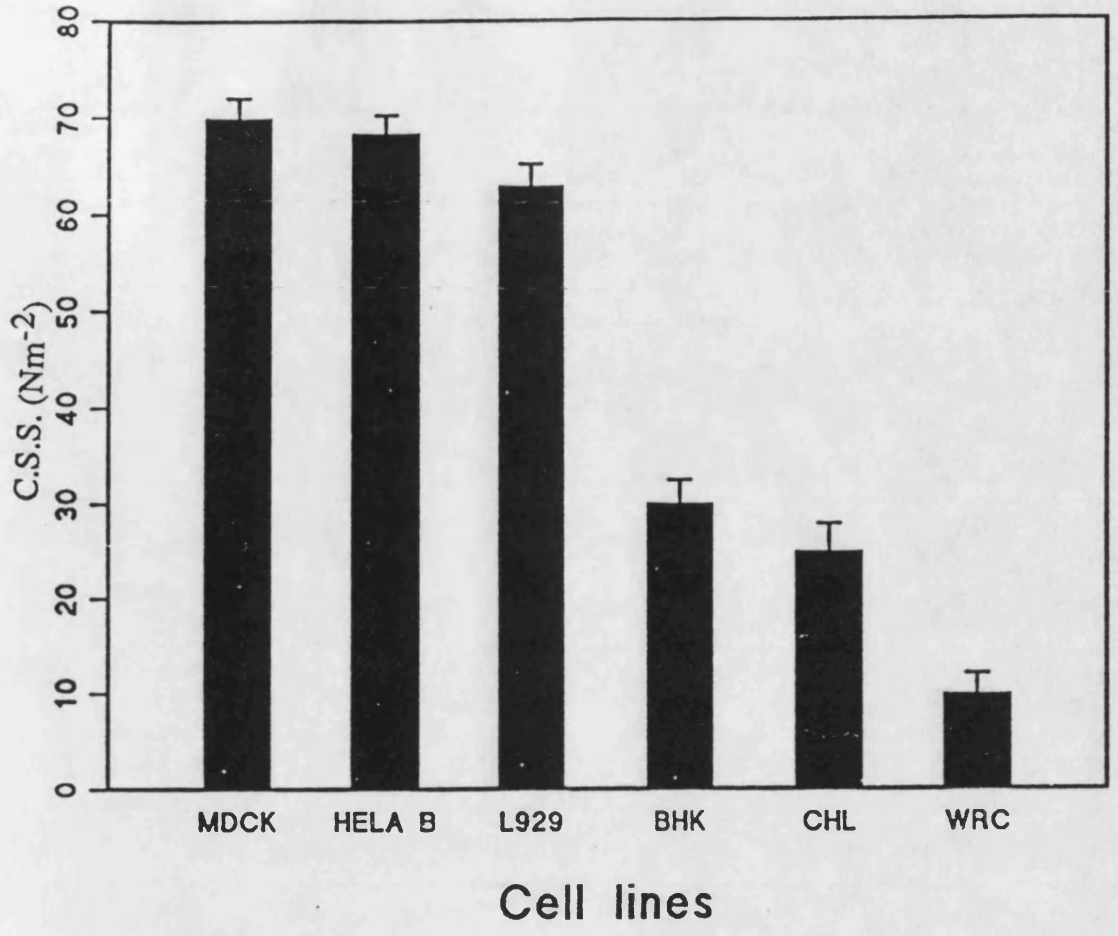


FIGURE 4.3

COMPARATIVE ADHESION STRENGTH OF DIFFERENT CELL LINES ON THE PLASTIC SUBSTRATUM.

MDCK, HELA B, L929, BHK, CHL, and Walker rat carcinoma cells (WRC) were grown on tissue culture grade plastic dishes (100mm) as outlined in materials and methods. The adhesion strength of these cells in terms of the critical shear stress (c.s.s.) of detachment was measured by using the Microflow chamber.

Each data point is the mean of 5 experiments each of which contains 20 measurements of c.s.s. of detachment. The error bars indicate the standard error of the mean. Further details may be found in the text.

in these figures the c.s.s. value for CHL cells unexpectedly dropped by 34%. Once again this emphasises the underlying differences in adhesion in different established cell lines.

A pleasing aspect of this adhesion analysis is the remarkably good reproducibility within a particular cell line. Moreover the c.s.s. value within a particular cell line was always (within a small standard deviation) close to that cell lines value whenever it was measured. At present the underlying mechanism of detachment is uncertain and equally unclear are the mechanisms which cause different surfaces to have different strengths. However, it is possible to make realistic speculations from the results obtained. These are described below.

It is clear from early work (Curtis, 1960; Baier & Dutton, 1969) that when a clean (virgin) surface is wetted with a solution containing protein(s) the first, very fast reaction is the adsorption of the protein onto the surface. Such adsorption leads to complete denaturation into a spread out 2 dimensional sheath. After a monomolecular layer is formed a subsequent build up of more protein layers occurs, the thickness of which depends on the nature of both the surface and the protein (Castillo et al, 1986). It is extremely difficult, if not impossible, to determine the resulting protein-substratum structure. However it is possible to adsorb radiolabelled proteins (chapter 7) and to calculate what the protein concentration is on the surface. Furthermore, by making assumptions about the size of the denatured protein one can predict with some confidence, how many layers there are adsorbed. Of coarse the situation is more complex if a mixture of proteins (e.g. serum) is adsorbed to the surface. It is probably a reasonable assumption that a cell does not, in fact, adhere to the surface but to the adsorbed protein layer on that surface. The resulting layer will also vary greatly in its structure varying widely from totally denatured to structurally intact proteins. Thus the cell receptors will have an enormous repertoire of ligands (surface proteins) to which they may bind. Of course a further complication is that the resulting receptor-ligand complexes will have widely different affinities for each other, which will be reflected in the final strength of adhesion.

Furthermore as stated earlier there must be a complicated signalling system between the cell- substratum interface and internal cellular mechanisms, such as protein synthesis. The hypothesis put forward in this thesis is that there is a signalling system which controls not only the secretion of proteins to their correct location within the cell- substratum interface but also determines the structural relationship between this interface, the extracellular matrix, the cell membrane, the cytoskeleton and metabolism. The signalling thus makes the cell adhere to the surface with a strength that seems to be genotypic though there is no direct evidence for this.

A discussion of the first step in the process now follows, that is the adsorption of the proteins to the substratum and the possible exchange of proteins within the adhesion interface. The latter comment implies that once the cell- substratum adhesion is made then there is a continuing import export of proteins between the cell and the adhesion interface. It must also be made clear that once the cells adhesion is formulated then the process does not stop there. At some stage in the cells growth cycle it will need to "round up" for division, a process which involves a complete change in the cell adhesion to the rounded up less adhesive mode.

In the present work the two surfaces or substrates of interest are glass and plastic.. The former is quite hydrophilic, contains no aromatic residues and is weakly ionic. The latter (styrene) is more hydrophobic, contains a large amount of aromatic molecules, and is probably more ionic than glass. That is the plastic is treated during manufacture with high voltage (plasma), which introduces -OH and COOH groups (Klebe et al, 1981). It is clear that the protein adsorption will be very different on both of these surfaces (e.g. plastic & glass).

Apart from controlling the amount of protein(s) which mediates cell adhesion, different substrates might be inflicting conformational changes in different proteins in different ways (Klebe et al, 1981; Van Oss et al, 1981). The receptors on different cells seem to be able to recognise these conformations with varying degree of affinity, thereby resulting in bonds of various affinity in each cell type. It seems that receptors of CHL cells make relatively weak bonds with protein(s) adsorbed on the

plastic compared to proteins adsorbed on the glass. One should keep in mind that these bonds might be responsible for sending signals to produce cells own proteins. Thus it is possible that the bonds that formed between receptors on CHL cell surface and proteins adsorbed on the plastic caused poor production of intracellular protein(s) and thus resulted in poor adhesion compared to the adhesion of the CHL cells on glass substratum. This idea gets support from the findings that extracellular matrix production is substrate dependent (Varani et al, 1989). Again this phenomena may depend on the responsiveness of different cells to the various substrata. This may be the reason that CHL cells showed poorer adhesion on plastic than glass. However the cellular and molecular basis of this phenomena cannot be confirmed at present.

4.4. AGEING IN RESPECT OF C.S.S. OF DETACHMENT.

Some cell lines also show ageing with respect to their critical shear value. For example when BHK cells were taken from liquid nitrogen storage and their growth maintained in $25cm^2$ flasks for a large number of passages the c.s.s. of detachment was found to slowly decrease from the original value of $25.9 \pm 3.9\ Nm^{-2}$ (figure 4.4). After 24 weeks from zero time the c.s.s. of detachment had dropped by 90% of the original value of the BHK cells. (figure 4.4). While the cell viability was found to be intact (98.8%) and not to decrease, there was a marked decrease in the adhesion strength of BHK cells as the passage number increased. On the other hand no difference in adhesion strength of L929 and Hela B cells was found from 0 time to 24 weeks (figure 4.4). The decrease of c.s.s. of detachment of BHK cells may be due to genetic instability of these cells which might easily alter many aspects of cell adhesion. These alterations includes perturbation of the biosynthesis of adhesion protein(s), integrin receptor(s) or an increase in proteolysis of normally expressed proteins. Moreover, the sensitivity of BHK cells to c.s.s. of detachment might reflect the altered communications between the adhesion proteins and the cell machinery. To

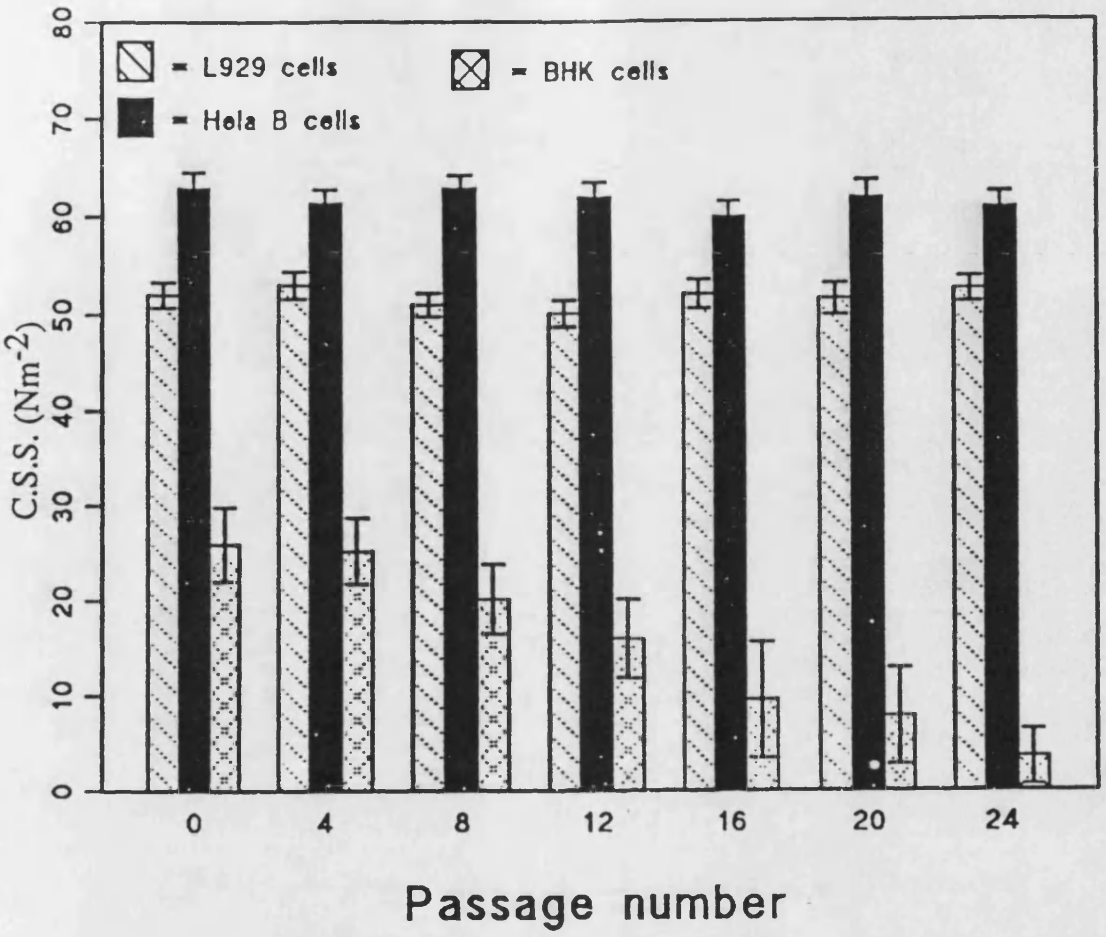


FIGURE 4.4

EFFECT OF AGEING ON THE C.S.S. OF DETACHMENT OF BHK, L929 AND HELA B CELLS.

BHK, L929 and Hela B cells were taken from liquid nitrogen and were passaged routinely after 3 days. The critical shear stress (c.s.s) of detachment of every cell line was measured weekly for 24 weeks. The ageing effect on the c.s.s. of detachment of BHK cells is shown in figure A. Figure B represents the comparison of ageing effect on the c.s.s. of detachment of BHK, L929 and Hela B cells. Each data point is the mean of 25 different determinations. The error bars represents the standard error of the mean. Further details may be found in the text.

explore these possibilities more information will be required on the biochemistry of extracellular and cytoskeletal proteins throughout their ageing period. Such information is currently not available.

At present an alternatively spliced fibronectin may be considered to be one cause for a change in adhesion because splicing may produce biologically active or inactive polypeptides of fibronectin. This speculation is supported by the study of Chandrasekhar et al, (1983). They demonstrated that human diploid fibronectin from young cells can make an old cell more adhesive, while old cell fibronectin was less effective in the adhesion of old cells. It was further suggested that the young cell fibronectin when compared to old cell fibronectin was more supportive to adhesion plaque formation. These observations may be implied similar to that which occurs in the ageing of BHK cells described above.

It is possible that due to defective fibronectin or other unknown molecules, the actin and microfilament fibres were not fully organised. It is also possible that the secretion system for endogenous extracellular matrix production was turned to a lower efficiency. These changes may have contributed to the basis for altered metabolism or altered biophysical properties. These altered properties of BHK cells may account for the decrease in their c.s.s. of detachment with increasing passage numbers.

4.5. CONCLUSION

In conclusion, a range of c.s.s. of detachment of various mammalian cell lines on glass and plastic was found. Every cell line has a specific and constant shear value with respect to the substratum. The tremendous versatility in adhesion strength of mammalian cell lines may become particularly evident with regard to the qualitative or quantitative expression of adhesion proteins and their receptors. Theoretical studies predicted the adhesion strength dependent on protein-receptor bond numbers. Whereas in the present work adhesion bonds are speculated to activate unknown second messenger which in turn regulate the activities of cytoskeletal proteins in

different cell lines in different fashions. Such mechanisms may cause each cell line to possess a different strength.

The Microflow chamber is validated in this cell adhesion research at receptor level and it has proved to be a useful tool for the characterization of cell-substratum interactions. For example, even though the qualitative attachment of different cell lines on the same or different surfaces may appear to be the same, the difference in c.s.s. will predict various proteins and class of receptors in each cell line. Which otherwise would have gone undetected. Thus a fundamental quantitative understanding of adhesion of cells to a substratum could lead us to understand the complexity of the cell adhesion phenomena.

These points are made in introducing the appropriate tools for understanding the mechanism of detachment under the effect of hydrodynamic force. It has been necessary to speculate about this phenomena because nothing is known about this aspect of cell adhesion. However, a start has been made to analyze the interaction that occur between a cell and its substratum. In this context the most obvious start point is with the adhesion molecules. That is, the adhesion proteins and the receptors involved in cell adhesion. These molecules are for convenience divided into three categories.

1 Exogeneously added as a mixture i.e. the serum component in the culture medium.

2 Exogeneously supplied purified adhesion proteins.

3 Endogenous adhesion molecules i.e. extracellular proteins and their receptors, produced by the cell.

The effect of each of these on the hydrodynamic detachment was examined. Thus the rest of this thesis will consist of the findings of this research.

CHAPTER 5
ROLE OF SERUM IN CELL ADHESION STRENGTH.

5.1. INTRODUCTION

It is generally accepted that the growth of virtually all types of cells in culture requires the presence of added serum in the culture medium. Such serum is a complex mixture and some of its components are directly involved in cell adhesion. Of course many serum components are as yet poorly characterised. Recently rapid progress has been made in the identification and characterisation of the proteins which mediate cell adhesion. Serum contains fibronectin, vitronectin, laminin, thrombospondin and many poorly studied proteins which mediate cell adhesion (Underwood & Bennet, 1989). However, there have also been reports that serum interferes with cell attachment and spreading (Witkowski & Brighton, 1972; Unhjem & Prydz, 1973). Curtis & Forrester (1984) demonstrated that components of serum that is alpha-1 antitrypsin and albumin both cause low adhesiveness of BHK cells to surfaces.

Considering these two contrary findings for the role of serum it was desirable to discover how serum effects cell adhesion. The approach made in the present chapter is based on attempts to understand what functions serum as a whole is serving in adhesion strength.

5.2. RESULTS & DISCUSSION

To define the quantitative role of serum in the adhesion strength the following strategies were adopted.

1 Firstly the c.s.s. of detachment of two cell lines i.e. L929 cells (fibroblasts) and Hela B cells (epithelial cells) was measured by simply varying the concentration of serum in the culture medium. The total absence of serum was also used as the starting point in the c.s.s. of detachment.

2 Secondly, great effort has been made to continuously grow L929 cells in serum free medium (Nutridoma medium) and to measure their adhesion strength.

3 The third and the last approach was to determine the effect of sera of different origins on the c.s.s. of detachment of L929, Hela B and Walker rat carcinoma cells. L929 and Hela B cells were selected because they are easy to grow and are taken as representatives of two different origins, i.e. fibroblasts and epithelial cells respectively. Walker rat carcinoma cells were selected because of very low adhesion strength (2.2±1.04 Nm^{-2} on glass and 10.2±1.5 Nm^{-2} on plastic) in the presence of horse serum. Therefore it was of interest to examine the effect of other sera on the adhesion strength of these cells. The details of the approaches (stated above) are given as follows.

5.2.1. EFFECT OF DIFFERENT CONCENTRATION OF FOETAL CALF SERUM ON THE ADHESION STRENGTH OF L929 & HELA B CELLS.

Here the experiments were based on the idea that one of the main functions of serum in adhesion studies is to provide a mixture of essential adhesion proteins e.g. fibronectin and vitronectin. These proteins are considered necessary for the attachment and spreading. It is clear that there are a large number of proteins present in the serum which may reinforce the stabilization of the initial attachment or *vice versa*. Prior to this study it has been impossible to measure cell adhesion quantitatively. That is why the quantitative role of serum has not been established. While now we can put a number to the adhesion strength of cells (with the development of Microflow chamber in present work) the first step is to construct an adhesion "response" curve for serum. To set this up L929 and Hela B cells were grown in 10%, 7.5%, 5%, 2.5%, 0.5% and 0% serum (v/v) in the culture medium supplemented with a good growth medium (Nutridoma 1%) and the c.s.s. of detachment was measured as illustrated in Materials & Methods.

The data are presented in the figures 5.1 & 5.2. It can be seen that there is a large decrease in adhesion strength of both cell lines between 0.5% to 0% serum containing medium. The adhesion strength of L929 cells remained constant when the concentration of serum was lowered from 10% to 0.5%. At 0% serum in the medium (supplemented with Nutridoma 1%) there was a sudden and statistically highly significant (p=0.0001) drop in the adhesion strength of L929 cells.

For Hela cells it is clear that there is a slow, but significant (p=0.0032) drop in the adhesion strength when the serum is lowered from 2.5 to 0.5% and then a very sudden loss of adhesion from 0.5% to 0% serum.

As it is seen in the figures 5.1 & 5.2, when serum was totally eliminated from the culture medium, a sudden drop in the adhesion strength of both cell lines was noted inspite of supplementation of good growth medium (Nutridoma 1 %). Thus it was logical to use culture medium in which serum should be diluted from 0.5% to 0.05%. The culture medium was also prepared without serum. This was done but without supplementing Nutridoma 1% (due to financial restrictions). In this condition L929 cells retained their adhesion when serum was diluted from 10% to 0.25%. These cells growing in culture medium containing 0.1% serum still showed a considerable adhesion strength ($40.5 \pm 5.5 Nm^{-2}$). However, without Nutridoma 1% medium, these cells did not survive in the culture medium containing 0.05% and 0% serum (table 5.1).

Hela B cells appeared more dependent on a good growth medium (Nutridoma 1%) as compared to L929 cells. That is Hela B cells did not survive in culture medium (without Nutridoma1%) containing less than 0.5% serum. Even at 0.5% serum (without Nutridoma) the adhesion strength ($24.0 \pm 5 Nm^{-2}$) of Hela B cells was just equivalent to the adhesion strength of these cells in serum free medium supplemented with Nutridoma medium ($23.0 \pm 8.5 Nm^{-2}$). These results indicate that a good growth medium which could fulfil the nutritional requirements of the cells was necessary not only for the stabilization of adhesion of cells but also for the survival of the l929 and Hela B cells in serum free medium.

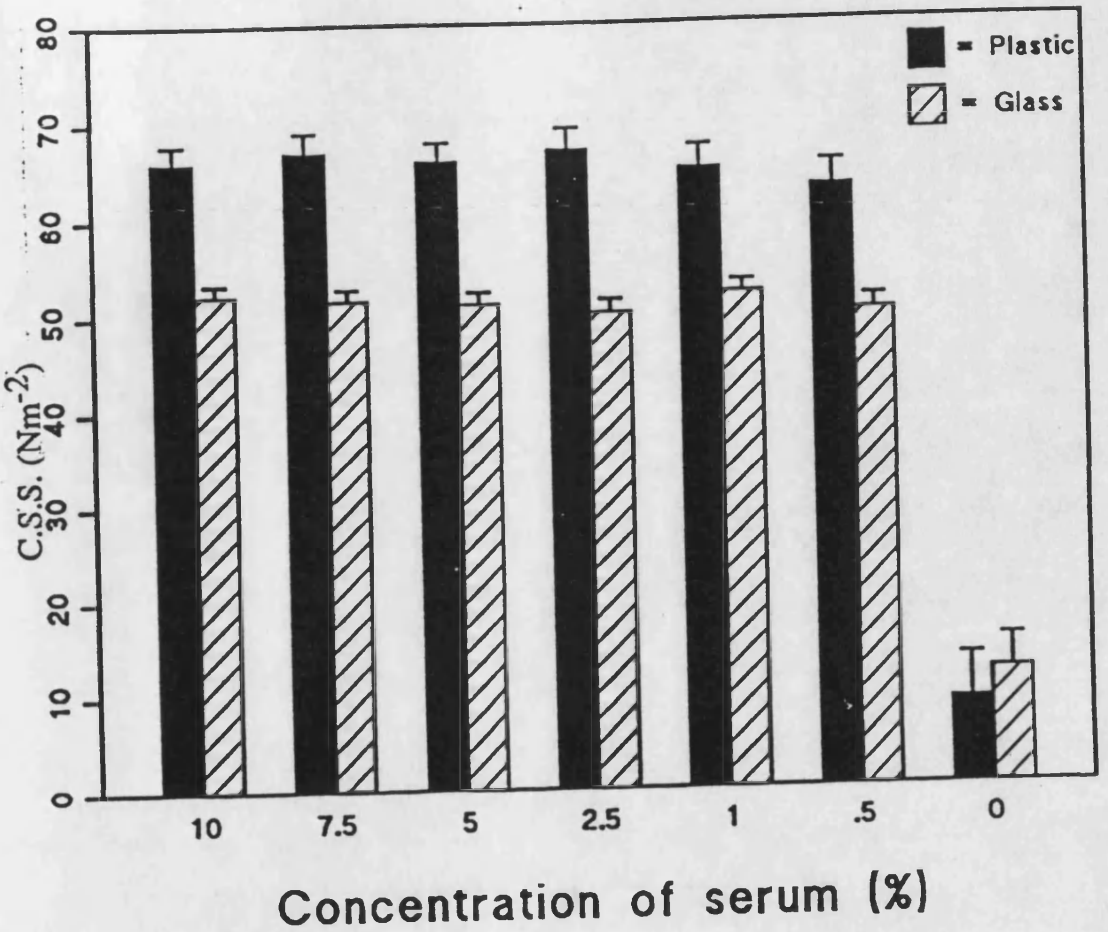


FIGURE 5.1

EFFECT OF VARIOUS CONCENTRATIONS OF SERUM ON THE ADHESION STRENGTH OF L929 CELLS ON PLASTIC OR GLASS SUBSTRATUM.

Sub confluent L929 cells were subcultured and maintained in the culture medium which was supplemented with 10 to 0% (v/v/) serum. These cultures (containing different concentrations of serum) were additionally supplemented with 1% Nutridoma. The adhesion strength of the L929 cells growing in these mediums is measured in terms of the critical shear stress (c.s.s.) of detachment.
Each data point represents 6 experiments in each of which 20 measurements were made. The error bars represent the standard error of the mean.
A t test indicates an insignificant difference ($p=0.25$) in the adhesion strength of cells growing in the medium containing 10% to .5% serum. Whereas this difference is highly significant ($p= 0.0001$) between .5% and 0% serum. Further details may be found in the text. The t test also indicates that in the absence of serum the difference between the adhesion strength of L929 cells on plastic and glass substratum is insignificant ($p=0.032$).

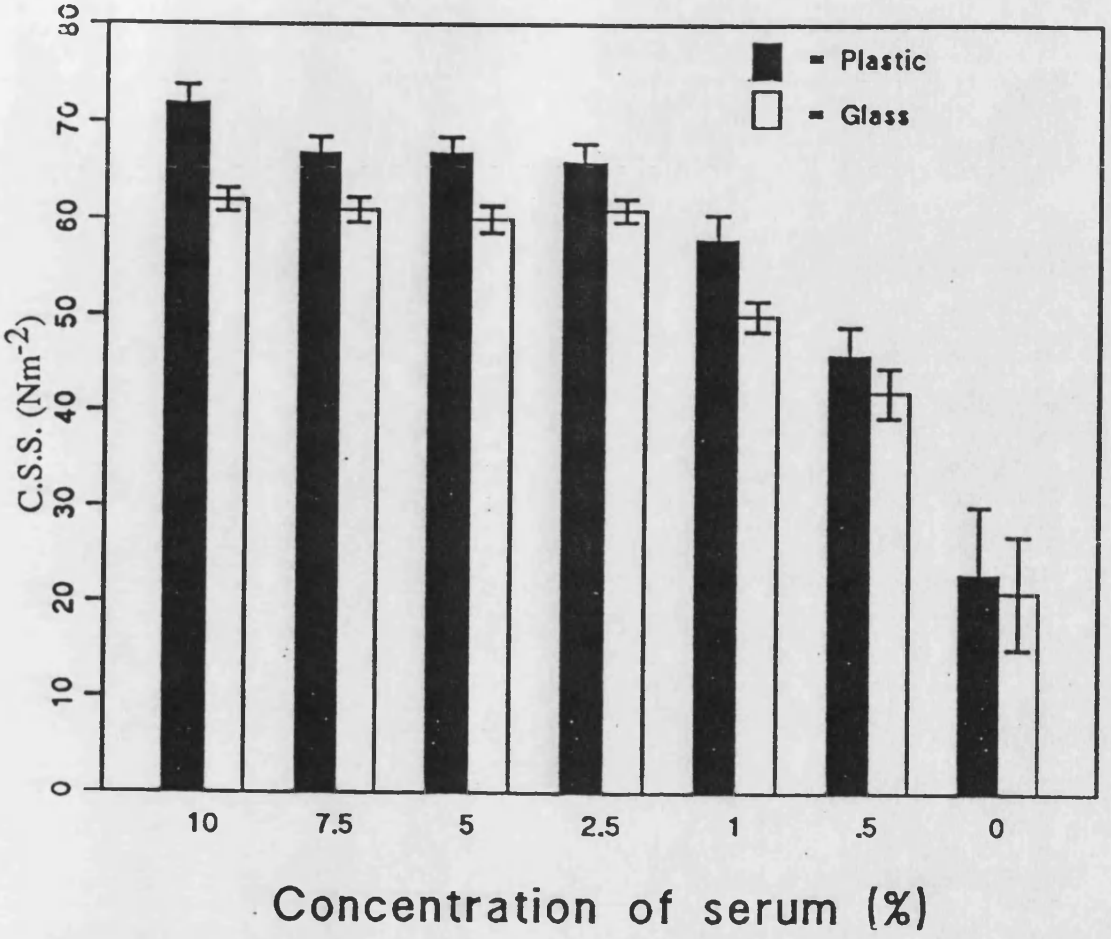


FIGURE 5.2

EFFECT OF VARIOUS CONCENTRATIONS OF SERUM ON THE ADHESION STRENGTH OF HELA B CELLS ON PLASTIC OR GLASS SUBSTRATUM.

Sub confluent Hela B cells were subcultured and maintained in the culture medium which was supplemented with 10% to 0%(v/v) serum. These culture (containing different concentration of serum) were additionally supplemented with 1% Nutridoma.

The adhesion strength of the Hela B cells growing in these mediums is measured in terms of the critical shear stress (c.s.s.) of detachment. Each data point represents 6 experiments in each of which 20 measurements were made. The error bars represents the standard error of the mean.

A t test indicates slightly insignificant (p=.003) difference among the adhesion strength of cells growing in the medium containing 7.5% to 2.5% serum. Whereas a highly significant (p=0.0001) difference was found from 2.5% to 0% serum. Further details may be found in the text. The t test also indicates that in the absence of the serum the difference in the adhesion strength of Hela B cells on glass and plastic is insignificant (p= 0.47).

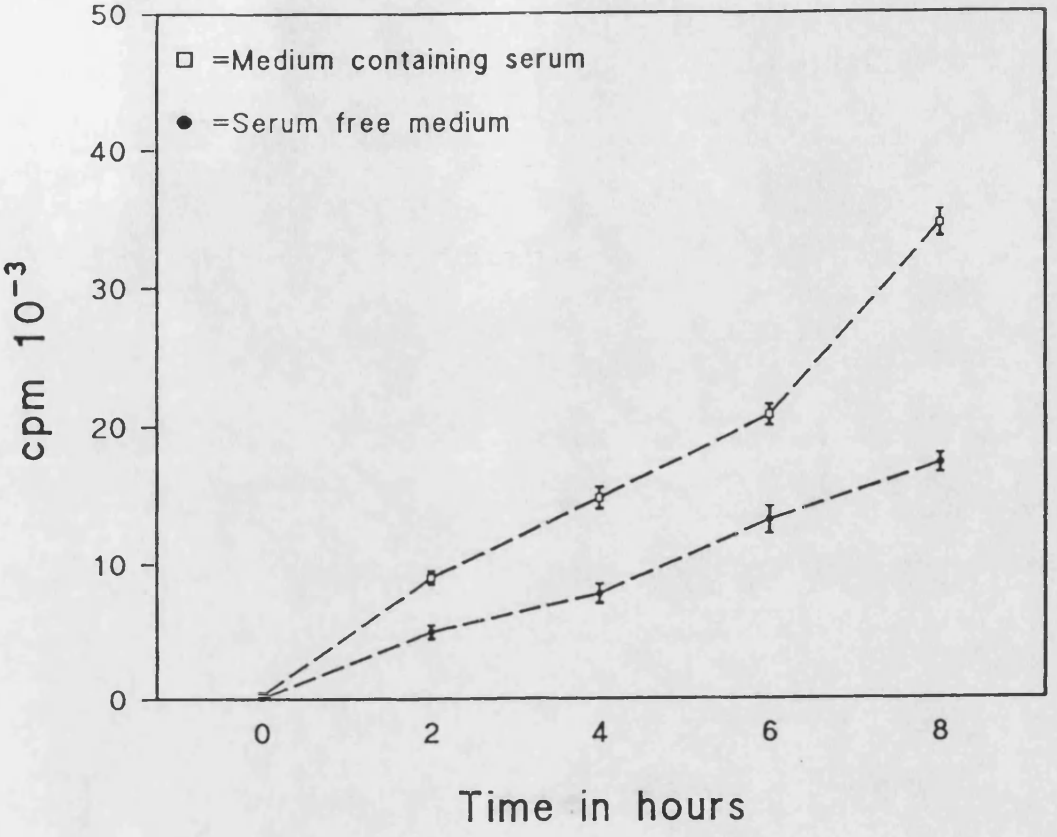


FIGURE 5.3

PROTEIN SYNTHESIS IN THE PRESENCE OF CULTURE MEDIUM WITH OR WITHOUT SERUM.

L929 cells were plated at a density of $5x10^4$ cells/cm^2 and were allowed to grow overnight in the culture medium containing serum. The old medium was replaced with the [^{35}S]-methionine labelled serum free medium or labelled culture medium containing serum. At different time intervals (indicated on the x-axis of the figure) the medium was carefully decanted and the cells were sequentially prepared for scintillation counting as illustrated in materials and methods.

Each data point represents 3 experiments in each of which every sample has been counted 3 times. The error bars indicate the standard error of the mean. Where error bar does not appear, it is smaller than the symbol. Further details of the experiment may be found in the text

Now the discussion of serum effect on the adhesion strength of these cells in presence of Nutridoma 1% follows. It is of interest that at 0% serum there is still a measurable and reproducible adhesion strength. However, in the absence of serum the cells are not growing in the sense that they do not go through cell division even a good growth medium was supplemented in the absence of added proteins. The effects of 0% serum will be discussed later, for the present, serum results are now discussed and conveniently divided into following major sections.

1. Effect of lowering the serum concentration from 10% to 0.5% on the adhesion strength of L929 cells.

2. Effect of lowering the concentration of serum from 10% to 0.5% on the adhesion strength of Hela B cells.

3 Growth of L929 cells in serum free media

4 Effect of 0% of serum on the both L929 and Hela B cells.

5 Possible mechanism by which serum exert its effects on the adhesion strengthening process.

5.2.1.1. EFFECT OF LOWERING THE SERUM CONCENTRATION FROM 10% TO 0.5% SERUM ON THE ADHESION STRENGTH OF L929 CELLS.

As seen in the figure5.1, in response to lowering the serum concentration from 10% to 0.5%, L929 cells appeared to maintain their normal adhesion strength. That is the difference between adhesion strength of L929 cells in presence of 10% to 0.5% serum is statistically insignificant. In fact it is equivalent to the adhesion strength of L929 cells in presence of 10% serum (53.0 Nm^{-2} on glass and 66.0 Nm^{-2} on plastic). The explanation of these results is given as below.

If serum is considered the only source of proteins for cell adhesion, it is likely that foetal calf serum even at 0.5% contains sufficient ligands to assign the final adhesion strength to L929 cells. There appears to be very strong support for this idea. That is foetal calf serum is reported to contain 300μg/ml of vitronectin and 30μg/ml

of fibronectin, which at 10% serum is sufficient to support cell adhesion (Hayman et al. 1985). If the concentration of fibronectin and vitronectin is simply diluted by lowering the serum concentration from 10% to 0.5%, the actual amount of fibronectin and vitronectin at 0.5% is thus 54.5ng/cm^2 and 545 ng/cm^2 respectively.

Now considering the co-distribution of fibronectin and vitronectin on the surface, while 545ng vitronectin/cm^2 is insufficient for attachment and spreading, 54.5ng fibronectin/cm^2 is adequate for attachment and subsequent stabilization of the cell adhesion. This point is made on the basis that the threshold level of vitronectin for attachment and spreading is quoted as 6.5μg/cm^2 (i.e.6545ng/cm^2 (Whateley & Knox, 1980) and in this work in chapter 7 it was found that 18ng/cm^2 of fibronectin is sufficient for L929 cells to attain final adhesion strength.

Grinnell and Feld (1982) and recently Underwood & Bennett (1989) found that adsorption of fibronectin to tissue culture plastic was minimum at 10% and maximum at 1% serum from the culture medium. The adsorption of vitronectin at 10% and 1% serum containing medium was exactly opposite to that of fibronectin (Underwood & Bennett, 1989). No reason for these observations is given in their studies, apart from that it was considered unknown phenomena. It may be that non specific proteins are hindering the adsorption of fibronectin by some means when culture medium containing 10% serum is used. Nevertheless, taken together these adsorption studies and findings of the present work (i.e. c.s.s. at 10% & 0.5% is the same) it can be suggested that at 10% foetal calf serum, it is vitronectin that is the effective molecule for adhesion strength of L929 cells and at 0.5% serum it is fibronectin that assumes this role.

Of course this interpretation is just speculation which is based on the protein adsorption studies carried out by others (Underwood & Bennett, 1989; Knox, 1984). However if this is true there must be biphasic phenomena in operation in the L929 cell adhesion strengthening process. This suggestion is consistent with the qualitative findings of Knox (1984) who suggested that at different serum concentrations spreading occurs *via* different mechanisms. That is different effectors of spreading

were proposed to become significant to a greater or lesser extent at higher or lower concentration of serum.

5.2.1.2. EFFECT OF LOWERING THE SERUM CONCENTRATION FROM 10% TO 0.5% ON THE ADHESION STRENGTH OF HELA B CELLS

As indicated in the figure 5.2 it is clear that there is a slow, but significant ($p=0.0032$) drop in the adhesion strength when the serum is lowered from 10% to 0.5% and thus a very sudden loss of adhesion in 0% serum (0% serum will be discussed later).

For the Hela B cells at 1% and 0.5% the c.s.s. of detachment was considerably reduced (15% and 30% respectively of the control value). Considering above different published reports it seems unlikely that the decreasing amount of adsorbed fibronectin is instrumental for decreasing the c.s.s. of detachment of Hela B cells, since sufficient fibronectin was shown to adsorb even at 0.5% serum. Further, there are reports that Hela B cells neither spread on a fibronectin substrate nor react with antibody specific for mammalian fibronectin receptor (Lu, et al, 1989). It is also reported that they do not produce fibronectin and fibronectin receptors (Becham & Jacobson, 1990). This indicates that Hela B cells use some protein(s) other than fibronectin for their adhesion. This protein (unknown) may be substantially diluted beyond its effective concentration at 1% & 0.5%.

It means that certain number of receptor-ligand bonds are needed to stimulate the events of adhesion strengthening phenomena. On the other hand at 1% & .5% serum L929 cells showed no decrease in adhesion strength perhaps the threshold level of adhesion bonds was achieved by using the adsorbed fibronectin. Whereas Hela B cells might be different in regard to their requirements for adhesion compared to L929 cells, nevertheless, these results suggested a significant role for serum (perhaps by providing adhesion protein(s)) in adhesion strengthening phenomena. This idea was further investigated by measuring the cell adhesion in the total absence of serum. For

this an attempt was made to grow L929 cells in serum free media. The difficulties faced are discussed below .

5.2.2. GROWTH OF L929 CELLS IN SERUM FREE MEDIUM

An effort was made to grow L929 cells in serum free media. The continuous growth of L929 cells was desired to see the effect on adhesion strength when adhesion proteins are totally excluded from the cell adhesion system. Moreover, it was tempted that once cells are adapted to serum free medium, different parameters involved in the cell adhesion could be examined. To fulfil the trophic requirements of the cells a medium was needed which provides nutritional elements (other than adhesion proteins) to the cells. To this end, L929 cells were grown in the serum free media (Nutridoma 1%) according to the instructions of the suppliers (Boehringer, 1989). Nutridoma was selected because it does not contain any adhesion proteins.

Firstly, L929 cells were inoculated directly in the serum free medium (materials & methods). The cells attached and remained attach to the culture vessel for 72 hours. Thereafter, they detached from the surface and were found floating in the culture medium. Their growth was determined by quantifying the number of cells at different intervals during 72 hours. Their number remained the same, indicating the cells did not grow at all. However, they were viable while they remained attached to the surface. After losing their adhesiveness (i.e. after 72 hours) the cells were found dead as was checked by trypan blue exclusion method. Therefore, it appeared that they needed some time to adapt to the new serum free medium. This was done according to the suppliers instructions. That is L929 cells were grown on serum free medium by gradually lowering the concentration of foetal calf serum from 10% to 0% (materials and methods).

For this purpose cryopreserved L929 cells were used in the earliest stage of their life. The cryopreserved cells maximise the genetic microavailability for adaptation of cells to the serum free media (Katsuta & Takaoka, 1973). This strategy was adapted because variations in each lot of serum cause an adaptation of the cells to

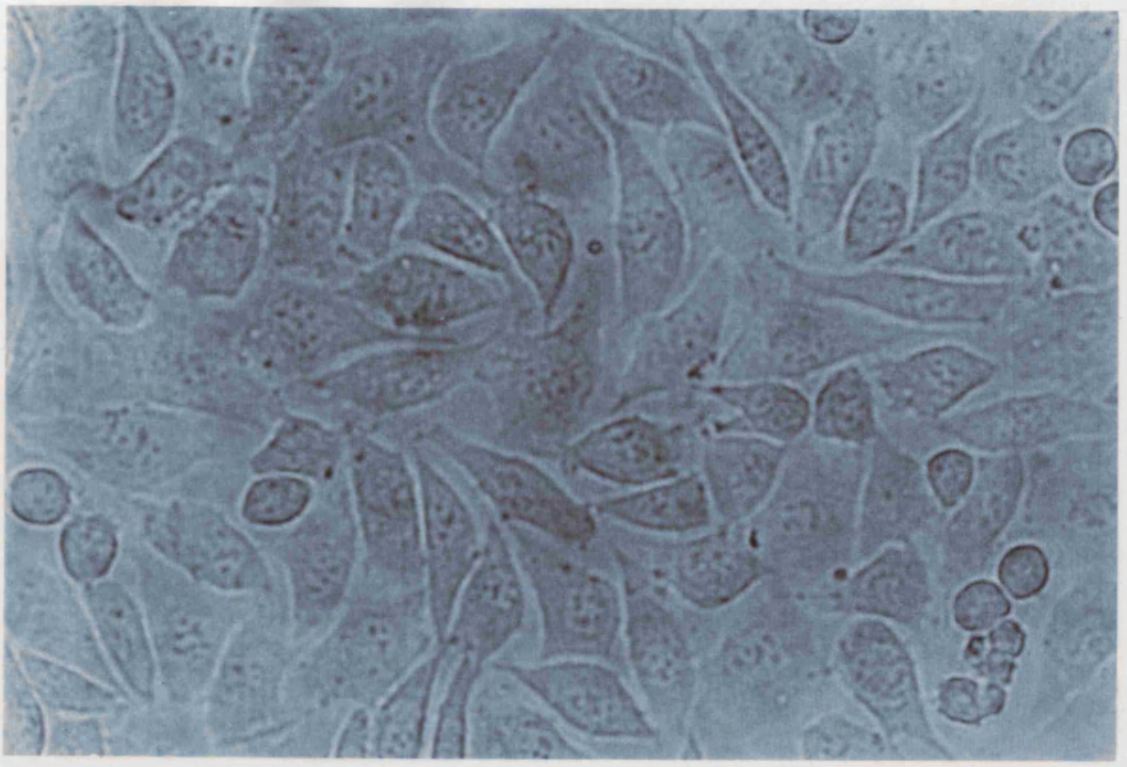
the specific serum. Thus cells which could adapt most quickly to the new conditions will become the prominent cells in the culture. There may not be a sub population of cells able to adapt quickly to a serum free environment. Considering this situation, it was logical to use the cells which were liquid nitrogen stored prior to use.
For the continuous growth of L929 cells in serum free medium the precautions which were outlined in suppliers information were carefully followed (Boehringer, 1989). The observations which were noted during the attempted adaptation of L929 cells are given as below.

1. The serum free derived cells were extremely sensitive to trypsin and only brief exposure to a little amount (0.01% w/v) compared to normal amount of trypsin (0.05%) was found sufficient. Moreover due to their sensitivity, they were handled gently.

2. Due to unknown reasons, bovine serum albumin, fraction V (0.1%) was helpful to maintain culture when supplemented into Nutridoma medium.

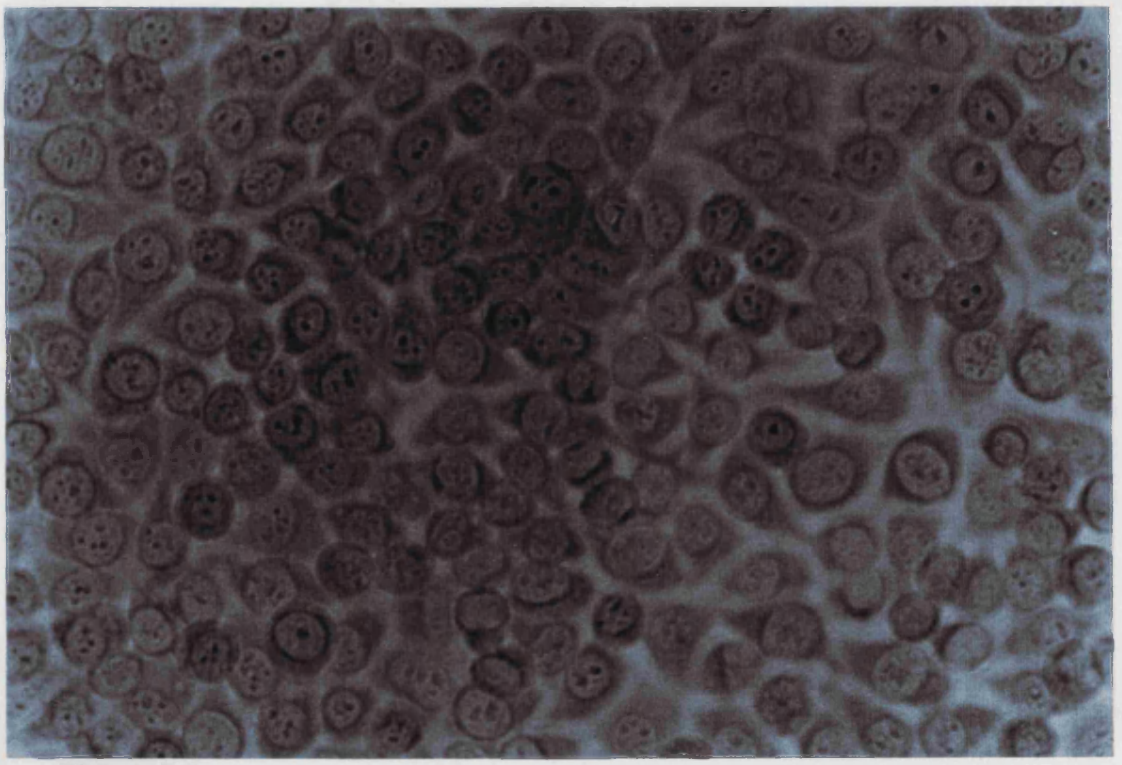
3. It is appropriate to maintain cell culture at high cell density ($3x10^5$ cells/ml), since only 25% cells were surviving at low inocula ($1x10^5$cells/ml).

Their growth curve was determined. While the doubling of L929 cells in serum was found to be 20 to 22 hours, the cells which were grown in the serum free medium doubled every 35 to 40 hours. However it is not out of place to mention that their doubling was not frequent and these cells could only be grown for 2 passages and in third passage they lost their adhesiveness. It took 3 weeks to bring these cells to this stage. Using the trypan blue exclusion method it was shown that these detached cells were viable (95%). The morphological studies of these cells showed that the cells were absolutely abnormal (picture 5.1 compared with picture 5.2 & 5.3). Their abnormality was also shown when their c.s.s. of detachment was measured. in first and second passages. In first passage their critical shear was 13.0



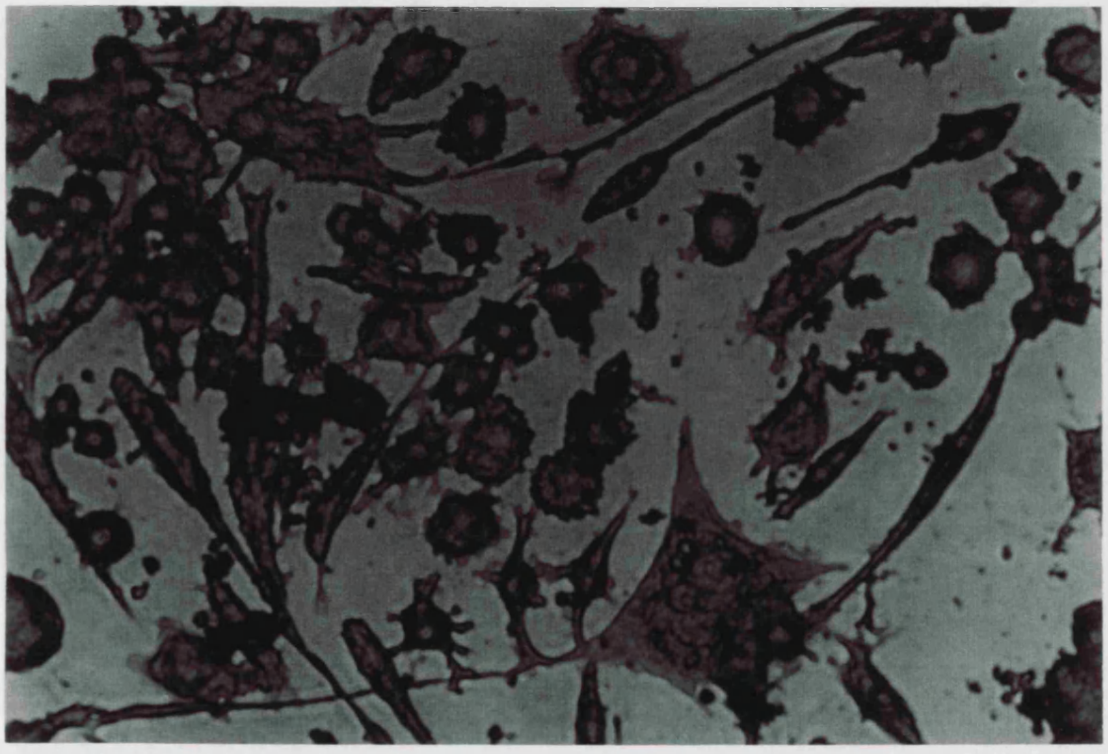
PICTURE 5.1

Normal L929 cells growing on the plastic substratum



Picture 5.2

Normal L929 cells growing in the serum free media



picture 5.3

Abnormal L929 cells growing in serum free media in their second passage

$\pm 5 Nm^{-2}$ (normal) and in second passage their critical shear value was 30.5 ± 18 Nm^{-2}(abnormal). The value of second passage was not stable (10-32 Nm^{-2}). The cells in their second passage repeatedly appeared abnormal as they were tried to adapt for 6 months. After 6 months extensive efforts it was concluded that continuous growth of L929 cells in Nutridoma was not possible. Therefore, it was decided to grow cells just for 24 hours or less (according to the requirements of the experiments) at the time of experiment, since at this time their c.s.s. of detachment is a constant value.

Thus in the connection of the effect of serum on the adhesion strength of L929 and Hela B cells, it was decided to check the effect of complete absence of serum proteins (adhesion) while cells had been growing for 24 hours in the serum free media. The effect of 0% serum on the adhesion strength of L929 and Hela B cells is discussed in the following section

5.2.3 THE EFFECT OF 0% SERUM (SERUM FREE MEDIA)

L929 and Hela B cells were trypsinized from their surface and trypsin was inactivated using soybean trypsin inhibitor (a 3xM stoichiometric excess). The cells were then inoculated simultaneously on plastic and glass substrata. To provide cells other essential nutrients (except adhesion proteins) in the absence of serum a good growth medium (Nutridoma 1%(v/v), see materials & methods) was supplemented for the culture medium. The c.s.s. of detachment was measured as illustrated in the methods (chapter 2). It is clear from the figure 5.1 & 5.2 that both cell lines showed a basal level of adhesion that is in 0% serum L929 cells have an adhesion strength (in terms of the c.s.s. of detachment) 9.1 ± 3 Nm^{-2} on plastic and 13.0$\pm$4.5 Nm^{-2} on glass. Hela B cells on the other hand have a strength of 16.0 ± 3.8 Nm^{-2} and 27.0 $\pm 7 Nm^{-2}$ on glass and plastic respectively (figure 5.1 & 5.2). Although, in the absence of serum adhesion strength on glass and plastic is different but it is statistically not significant. However, according to the trend, in the absence of serum, L929 cells comparatively stick better to the glass than plastic. Whereas Hela B cells attached better on plastic.

It is likely that this difference is due to cell type difference i.e. L929 are fibroblastic and Hela B are epithelial cells. However, what ever is the case, in both cell lines (on plastic and glass), the adhesion strength is substantially decreased (figure 5.1 & 5.2). It was of interest to note that in the absence of serum as compared to in its presence, the c.s.s. of detachment of both cell lines was obtained with unexpectedly large standard deviation. This observation might reflect the variation which may exit at sub-population level within the same cell line (Hurum et al, 1982; Connor et al, 1983)

5.2.3.1 POSSIBLE MECHANISMS OF SERUM EFFECT

5.2.3.1.1. ADHESION PROTEINS IN THE SERUM

The decreased critical shear in the absence of serum may be due to deficiency of adhesion proteins since considerably lower protein synthesis was observed in this case compared to the protein synthesis in the presence of serum (figure 5.3). Thus serum may be acting as a stimulant for protein synthesis and secretion. Since this stimulant was no longer provided, the cells might fail to carry on full scale adhesion protein synthesis and subsequently a low amount of protein deposition on the substratum took place. This is just speculation because adsorbed proteins were not analyzed in the present work. However, if it is the case, a minimum number of bonds might be formed to give the basal c.s.s. of $9.1 Nm^{-2}$ (in case of L929 cells) and $27.0 Nm^{-2}$ (in case of Hela B cells). Therefore, it is possible that in the absence of serum, the number of adhesion bonds underneath the cells responsible for final adhesion strength remained well below than required for this complex phenomena. It is not clear whether in the absence of serum the synthesis and secretion of all proteins decreased in a coordinated fashion or whether certain proteins are depressed preferentially. Thus it is my feeling that serum is providing vital ligands (proteins) for the cell surface receptors. These ligands bind with their receptors and

send fully developed informational signals (perhaps for protein synthesis) to the interior of cells so that cells attain final adhesion strength. In fact in the absence of actual adhesion proteins, serum has been considered necessary for cytoskeletal orgnisation (i.e. binding of actin to the talin and vinculin which in turn bind to the receptors) and adhesion plaque formation. These structures may play a role in the stabilization of adhesion process (Burridge et al, 1988). If proteins are provided then even when serum is absent cells achieve their final adhesion strength. (findings of chapter 7).

5.2.3.1.2. PROTEOLYSIS

In the absence of adhesion proteins in the culture medium enhanced secretion of hydrolases has been observed (Werb et al, 1989). These enzymes may be acting on receptors, adhesion proteins or cytoskeletal proteins. The end result will be fewer bonds and subsequently lower adhesion strength. Together, deficiency of adhesion proteins and enhanced secretion of hydrolases might render the cells less adhesive in the absence of serum in the culture medium.

5.2.4. EFFECT OF SERA OF DIFFERENT ORIGIN ON THE ADHESION STRENGTH OF L929, HELA B AND WALKER RAT CARCINOMA CELLS

After examining the different aspects of the role of foetal calf serum on two very different cell types, it was of interest to know whether or not sera from different species exerted similar or different effect on the adhesion strength of cells. Particularly, considering the c.s.s. of detachment of Walker rat carcinoma when grown in horse serum (figure 4.1 & 5.4), it was necessary to examine the effect of foetal calf serum on the adhesion strength of walker rat carcinoma. Also it was necessary to examine the effect of horse serum on the c.s.s. of detachment of L929 and Hela B cells. Therefore experiments were carried out to examine the

Conc. of serum	L929 C.S.S.(Nm^{-2})	Hela C.S.S.(Nm^{-2})
10.0%	63.8 ±2.5	66.5 ±2.8
7.5%	64 4±1.5	65 1±2.3
5.0%	63 0±2.7	65 0±1.7
2.5%	64 2±2.4	64.5 ±2
1.25%	63 3±2.1	45 5±3.5
0.5%	62 7±2.5	23 0±8.5
0.25%	63 6±2.8	0 (not suvived)
0.1%	40 5±5.5	0 (not survived)
0.05%	0 (not survived)	0 (not survived)
0%	0 (not survived)	0 (not survived)

TABLE 5.1

L929 and Hela B cells were grown in the medium with different concentrations of serum (without Nutridoma 1%). The critical shear stress of detachment of these cells was measured as illustrated in materials and methods. Each value in the table is the mean of 20 determinations.

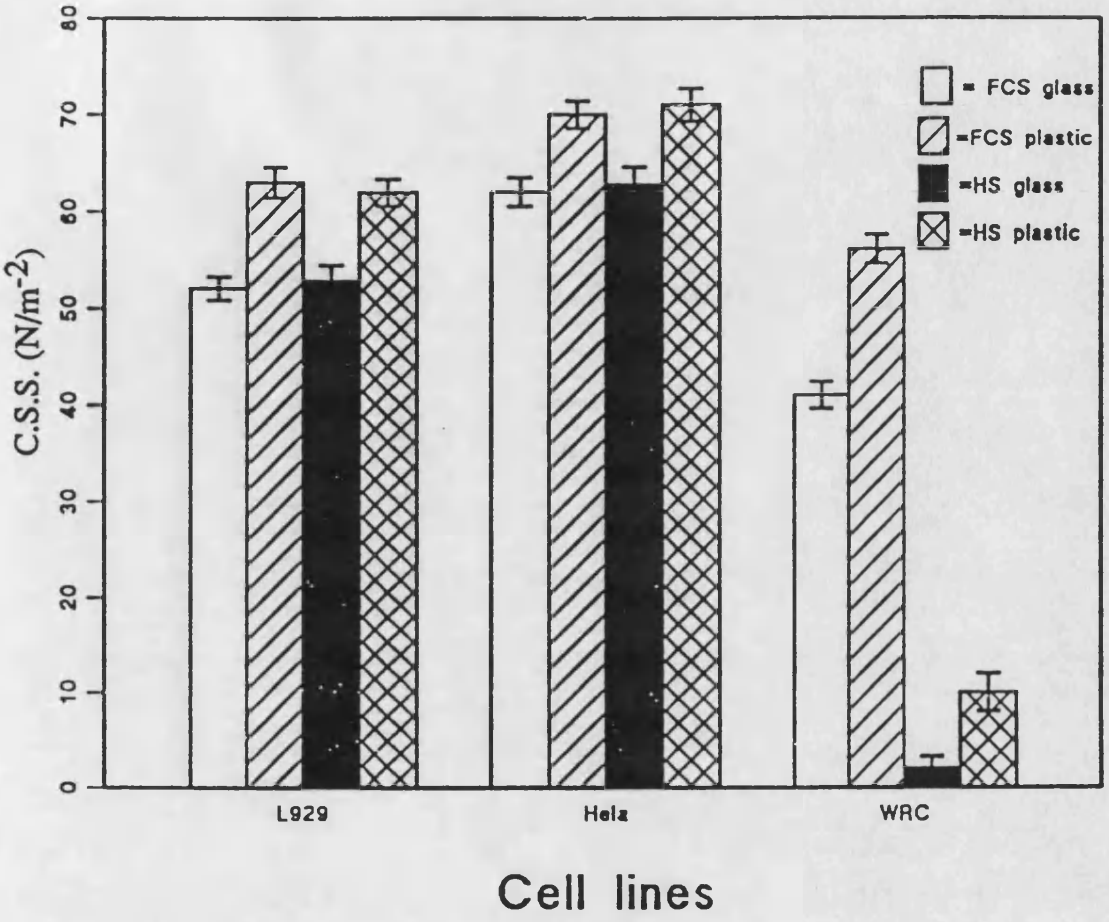


FIGURE 5.4

EFFECT OF FOETAL CALF (FCS) AND HORSE SERUM (HS) ON THE ADHESION STRENGTH OF L929, HELA B AND WALKER RAT CARCINOMA CELLS (WRC).

L929, Hela B and Walker rat carcinoma cells were seeded in the medium supplemented with horse serum (HS) or fetal calf serum (FCS) onto glass or plastic substratum. The figure represents the adhesion strength of these cells in terms of the critical shear stress (c.s.s). of detachment.
Each data point is the mean of 5 different experiments. Each of which contains 20 measurements of c.s.s. of detachment.
The error bars indicate the standard error of the mean. Further details may be found in the text.

responsiveness of L929, Hela B cells and Walker rat carcinoma to both horse and foetal calf serum. The results are presented as below.

L929, Hela B and Walker rat carcinoma cells were grown in foetal calf serum or horse serum as illustrated in materials and methods. The c.s.s. of detachment of each cell line was measured according to the detachment assay described in materials and methods. The c.s.s. of detachment of L929 and Hela B cells, in presence of horse and fetal calf serum was not significantly different (figure 5.4). That is for example the c.s.s. of detachment of L929 cells in presence of foetal calf and horse serum on plastic was 66.0± 2.3Nm^{-2} and 65.2±2.5 Nm^{-2} respectively (figure 5.4). Similarly the c.s.s. of detachment of Hela B in presence of foetal calf serum on glass and plastic was not significantly different than that in the presence of horse serum (figure 5.4). Surprisingly this was not the case for Walker rat carcinoma cells. In fact there was a dramatic increase in the c.s.s. of detachment of this cell line from both plastic and glass in response to foetal calf serum (40.0Nm^{-2} and 50.1Nm^{-2} on glass and plastic) compared to that of horse serum (2.2Nm^{-1} on glass and 10.5Nm^{-2} on plastic).

This horse serum–foetal calf serum difference is very exciting, though difficult to interpret in detail. Firstly, it shows that Walker rat carcinoma cells have a stable adhesion constant in each serum. For example in horse serum the c.s.s. of detachment of walker rat carcinoma cells is 2.2Nm^{-2} and in fetal calf serum it is 40.0Nm^{-2}. Furthermore these very widely different critical shear values are not reflected by measurable difference in growth rates. From an applied point of view this is further good evidence that the adhesion strength of a cell line is a suitable value that could be used as a quality control parameter. It is not easy to see how this adhesion strength difference arises. Ehrismann et al (1982) characterized the major adhesion protein "fibronectin" in horse serum and showed that in respect to its structure and functions it was similar to foetal calf fibronectin. So the different response is unlikely to be due to different fibronectins in horse and foetal calf serum. It is also unlikely that the structure of vitronectins is different in horse and foetal calf serum, since overall this is

also a quite highly conserved protein. However this difference can be attributed to the following speculations

1 Involvement of protein(s) other than fibronectin and vitronectin

2 Horse vitronectin

(i) Glycosylation

(ii) Different receptors of vitronectin

(ii) Proteolysis

3 Transferrin

Now these points will be discussed individually as below.

5.2.4.1 PROTEINS OTHER THAN FIBRONECTIN AND VITRONECTIN

It is likely that the differences lie in the many other proteins present. If this is the case, the unknown adhesion protein(s) present in the foetal calf and missing from the horse serum may have a little significance for Hela B and L929 cells, whereas they may be crucial for strengthening the adhesion of Walker rat carcinoma cells. It is also possible that these unknown adhesion proteins may be present in abundance in horse serum and could have low affinity toward receptors on the Walker rat carcinoma cell surface. On the other hand , foetal calf serum may contain proteins with high affinities for Walker rat cell surface receptors. These results indicate that Walker rat cells may attach and spread in similar way to Hela B and L929 cells but they probably attain their strength under entirely different mechanisms. The other possible mechanisms involved in the adhesion strengthening phenomena of Walker rat and other cells is given below.

5.2.4.2. HORSE VITRONECTIN

Kitagaki-Ogawa et al (1990) published results that vitronectin of different serums may show variations with respect to its glycosylation sites . The most remarkable difference was found in D-galactosamine content, where horse vitronectin

contained one residue and bovine vitronectin contained 8 residues of this molecule. In addition, other quantities of carbohydrate e.g. mannose and galactose on horse serum vitronectin are considerably lower compared to bovine vitronectin. It is known that carbohydrate generally stabilizes the protein structure and may be a protection against proteolysis (Rademacher et al, 1988). Therefore, it may be suggested that vitronectin in horse serum mediates attachment and spreading but due to lack of carbohydrate content was more prone to proteolysis than calf serum vitronectin. It may well be that extracellular proteolysis of vitronectin disturbed the vitronectin-integrin-cytoskeleton complex and affected the formation of adhesion plaques, thereby, reducing the adhesion strength of Walker rat carcinoma cells. It could be argued that if this is the case then horse vitronectin should be equally target of proteolysis in case of L929 and Hela B cells. In this regard the following factors must be considered.

Firstly, the proteolytic activity may be different in Walker rat carcinoma cells and other two cell types. Secondly, horse serum vitronectin may be equally facing proteolysis in L929 and Hela B cells but fibronectin and/or some other proteins are still there to compensate for this loss. If it is true then Walker rat carcinoma cells may be using vitronectin for its attachment and subsequent adhesion strengthening phenomena. While it can be speculated that different vitronectins show different binding affinities for the cell surface receptors, it can also be suggested that highly glycosylated foetal calf vitronectin may be providing more resistance to proteolysis, thereby , increasing adhesion strength of Walker rat carcinoma cells.

Apart from adhesion proteins horse serum may be deficient, quantitatively or qualitatively in one or more components essential for the adhesion strength of Walker rat carcinoma cells and non essential for L929 and Hela B cells. These components may include the molecules such as transferrin and growth hormones which are described below.

5.2.4.3. GROWTH HORMONES

Some hormones can alter the expression or affinity of receptors for proteins and in this way might be expected to affect cell adhesion. For example, for some cell types it has been established that the amount of fibronectin they produce can be modulated by glucocorticoids (Oliver et al, 1983), cyclic AMP (Dean et al, 1987) and epidermal growth factors (Baltt et al, 1988). These hormones are effective at extremely low concentrations (pico molar basis) which might vary between horse and foetal calf serum and thus would make difference between the effectiveness of horse and foetal calf serum. If it is the case they are much more important for Walker rat carcinoma cells but not for L929 and Hela B cells.

5.2.4.4. TRANSFERRIN

In the hormone category, there is a protein called transferrin. As with insulin, virtually every cell type examined has been found to respond to transferrin in serum free media (Barens and Sato, 1980). Iron saturated transferrin is essential for maintenance of cells in culture. The stimulatory activity of transferrin may also be due to the binding of this molecules to other metal ions which are toxic and which may be present in the culture medium (Mather and Sato, 1979). It is possible that each cell type has a different requirement for transferrin to cause effective adhesion as was shown for the growth of some human carcinoma cell lines (Allegra & Lippman, 1978). Recently it has been shown that proliferation of chick embryo neuroblast growth in the presence of horse serum requires exogenous transferrin (Barakatwalter et al, 1991). Since the growth of Walker rat carcinoma cells in horse serum is perfect, transferrin is either irrelevant to the growth of these cells or it may only be affecting their adhesion strength.

5.3 CONCLUSION

In conclusion, adhesion proteins which are present in the serum are responsible for the adhesion strength of cells. In 0% serum containing culture

medium, cells remained confined in first phase. That is they never gain their final adhesion strength. Moreover, the results presented in this chapter show that spreading is one aspect of cell adhesion but does not represent the final adhesion strength as was found in the case of Walker rat carcinoma cells. That is they spread equally well in the presence of horse and foetal calf serum but they are unable to gain their final adhesion strength in presence of horse serum. Thus in presence of horse serum they never entered into the second phase of cell adhesion (final adhesion strength). Whereas in presence of foetal calf serum they go through from first (attachment +/- spreading) to second phase (final adhesion strength).

While these findings and their interpretations are valuable information for the future research, the emphasis on these findings must be that the adhesion value of a cell line is constant value under constant conditions. It is not out of place to mention that the main objective of this study was to check the potential of the Microflow chamber (developed in the present work) for evaluating the adhesion strength in different type of environments. It is very pleasing about this device that it is able to take account of the environmental adhesion potentials of the system. Therefore, now it is possible that the data obtained by the Microflow chamber can be interpreted according to the requirements of the work (research, clinical and biotechnological).

From the speculations in the previous chapter that specific adhesion protein(s) are responsible for cell adhesion strengthening phenomena, created the immediate thought that they might be doing so by binding with the integrin receptors. The adhesion proteins might be sending signals to the interior of cells via integrin receptors. This is only speculation because nothing is known about it. Nonetheless this is not the subject of this study, rather this speculation can be considered as a suggestion for future work. Thus the attention was focused on the protein receptor complex. It was thought that if protein-receptor bonds were involved, by blocking receptors with some agents, these bonds can be prevented from forming and in this way the adhesion strength of a cell can be reduced or abolished completely. One approach was to use small peptides (e.g.RGDS) which are known to have the active

binding sites of fibronectin and other adhesion proteins (see chapter 1). These ought to bind to the integrin receptors thus competing out the respective protein(s)–receptor(s) binding. and adhesion strength of a cell can be purturbed. The following chapter is based on this strategy

CHAPTER 6

INHIBITION OF ADHESION STRENGTH IN RESPONSE TO RGDS(Arg- Gly-Asp-Ser) & YIGSR (Tyr-Ile-Gly-Ser-Asp).

6.1. INTRODUCTION

Synthetic peptides containing the sequences RGDS and YIGSR have been shown to inhibit the adhesion of various cells to several extracellular matrix protein(s) (Saiki et al, 1989). While the former inhibits adhesion mediated by fibronectin, vitronectin, laminin, collagen, the later specifically inhibits the laminin mediated cell adhesion (Skubitz et al, 1990). The RGDS sequence was first mapped as the main interaction site for the cell surface fibronectin binding receptor $\alpha 5\beta 1$ (Pytela et al, 1985b). Since then RGDS has been used to inhibit the function of the $\alpha 5\beta 1$ and other integrins in several experimental models (Ruoslahti & Giancotti, 1989; Reinbolt et al, 1990)

The YIGSR a laminin derived peptide has also been reported to bind to a 67Kd laminin receptor(Terrenova et al,1983). In the present study, RGDS and YIGSR were used to block the receptors and the resultant effects on the adhesion strength of L929 and HELA B cells were examined. It was in mind that if prior to the detachment measurement, the fibronectin active binding site peptide RGDS was added, a measurable decrease in adhesion strength might be seen.

6.2. RESULTS & DISCUSSION

6.2.1. EFFECT OF RGDS ON THE ADHESION STRENGTH OF L929 CELLS

The effects of RGDS on the critical shear stress of detachment of L929 cells from glass and plastic substrata were examined by incubating the cells with this peptide in varying concentration for 24 hours.

As seen in figure (6.1), the depression of adhesion in presence of RGDS to some extent was concentration dependent, however, by increasing the concentration from 7.5μg/ml to 15μg/ml, the adhesion strength was not further perturbed. The maximum reduction noted on glass and plastic substrata was 44% and 41% of the control values respectively The effect due to RGDS did not appear to be due to toxicity rather, it seemed to be quite specific. This specificity was demonstrated by the fact that the control peptide i.e. YIGSR (materials & methods) had no observable effect on the adhesion of L929 cells. Lack of toxicity was also evidenced by the observation that no cell death was detected by the trypan blue exclusion method, where the RGDS was present in the growth medium of the cells.

As seen in figure 6.1 the reduction of adhesion strength was only partial at the maximum concentration of RGDS used in this study. The reason may be that the concentration used in this study was far lower than the amount of peptide used in previous studies published by the other workers (Ylänne, 1990). That is the maximum concentration utilized in this study was 15μg/ml compared to 1000μg or more/ml in other studies (Hautanen et al, 1989 and Massia & Hubble, 1990). However one must make distinction between the effect of RGDS either on attachment of the cells or on the strength of cell adhesion. Here in this study it was never the aim to stop the cells attaching to the substratum.

It is not clear, at the present time, how the adhesion functions are perturbed by this peptide. It is likely that vitronectin and fibronectin are both implicated because both of these adhesion proteins are present in the serum for growing these cells. Moreover Hayman et al (1985) have shown that serum proteins play a role in cell adhesion. The reduction in adhesion strength seen in the figure (6.1) is significant when one realizes that the RGDS interaction is, by no means the only receptor–ligand bindings present (Farsi et al,1985 and McCarthy et al, 1990). Even within fibronectin there are other adhesion domains (Komoriya et al, 1991). Thus the partial reduction of adhesion strength either may be due to RGDS independent molecules or to domains

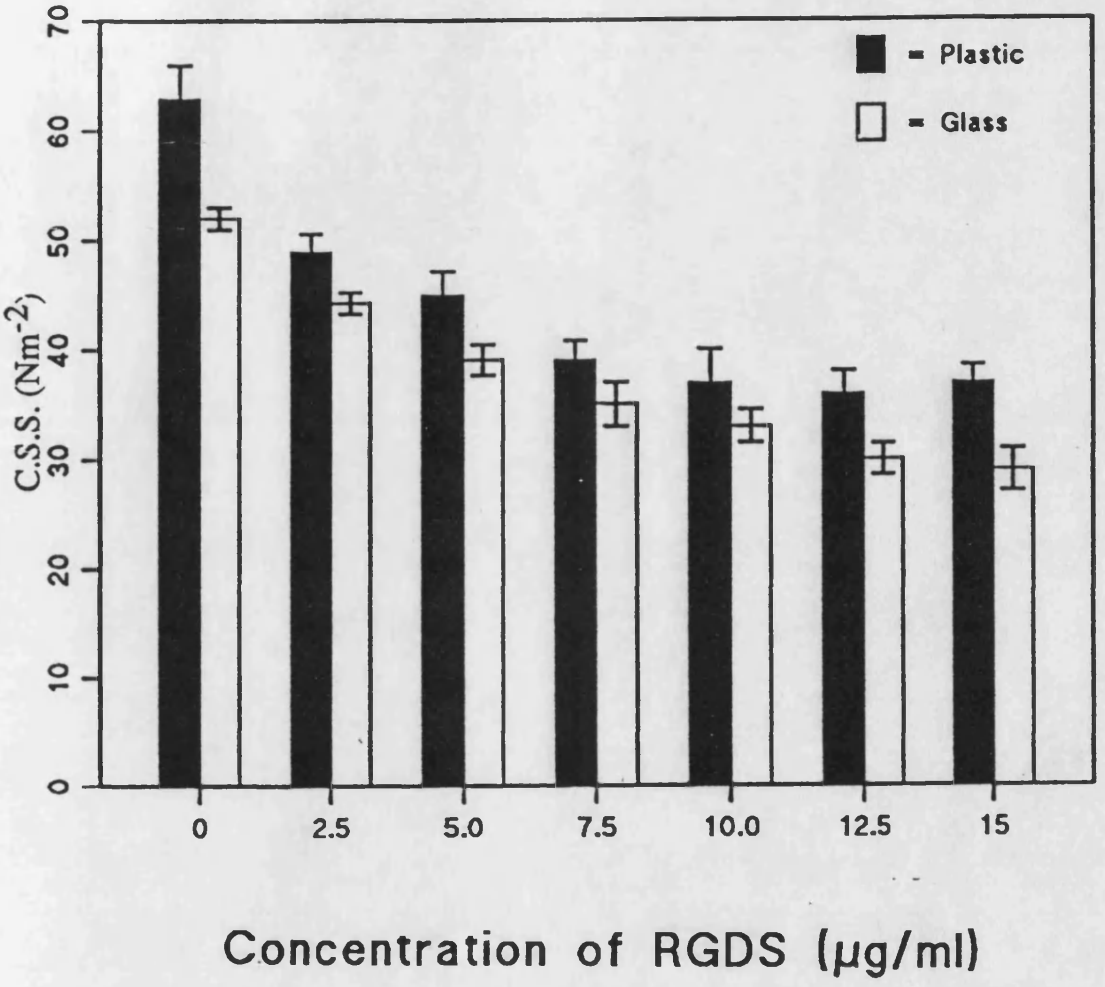


FIGURE 6.1

PERTURBATION OF ADHESION STRENGTH OF L929 CELLS IN RESPONSE TO RGDS (arg-gly-asp-ser).

L929 cells were plated on glass or plastic substratum with or without RGDS peptide at the concentrations indicated on the x-axis of the above figure. Adhesion strength of these cells in terms of the critical shear stress (c.s.s.) of detachment was measured by using the Microflow chamber as described in materials and methods.

Each data point represents 3 experiments, each of which contains 20 measurements of c.s.s. in response to different concentrations of RGDS peptide. The error bars indicate the standard error of the mean.

A t test indicates that the depression of adhesion strength of L929 cells even at lower concentrations (2.5µg/ml) of RGDS is highly significant ($p=0.0001$). Further details may be found in the text.

other than RGDS (arg-gly-asp-ser).It is also quite likely that RGDS binding was less than 100%. That is, it is a competitive reversible binding event whose quantitative affinities or disassociation constant is not known. In the present study in correspondence to maximum concentration (15μg/ml) $1.8x10^{16}$ molecules of RGDS/ml were calculated, where as maximum number of receptors on the cell surface are estimated as $1x\ 10^{7}$ (Codongo et al, 1987). The total volume in the adhesion assay plate ($55cm^2$) was 20 ml thus the total number of RGDS molecules per plate can be calculated as $1.8x10^{16}$/ml x 20 = $3.6x10^{17}$/plate. Whereas total number of cells in the plate ($55cm^2$) were $4x10^{6}$, therefore, number of receptors per plate were $4x10^{6}$ x 10^{7}receptors/cell = $4x10^{13}$/plate. From this calculation it seems that sufficient molecules of RGDS were present to compete out receptor-ligand binding but one must keep in mind that it is competing with much larger molecules of both serum and secreted proteins.

One body of evidence suggested that the treatment of mouse 3T3 fibroblasts with RGDS not only perturbed binding of adhesion proteins to their receptors but also induced disassociation of cytoskeletal proteins from the sites of adhesion plaques (Stickel & Wang, 1988) The cytoskeletal proteins and adhesion plaques both are considered necessary for the stabilization of cell adhesion (Burridge et al, 1988). Thus it seems likely that RGDS used in present study affected adhesion strength by disturbing the adhesion plaques or by interfering in the formation of adhesion plaques. As it is also possible that the binding of RGDS with the receptors might have enhanced the expression of proteases which caused the reduction of adhesion strength (Werb et al, 1989)

In response to RGDS the partial reduction in adhesion strength of L929 cells can be explained from another point of view. That is all the previous published studies with the RGDS were carried out for short time periods (Pierschbacher & Ruoslahti, 1987; Ruoslahti & Pierschbacher, 1987). This is the first study to date in which functions of RGDS are determined for a longer period in serum containing medium. Thus similar effects noted in response to all concentrations at or above 7.5μg/ml may

be suggesting the reversibility of RGDS-receptor interaction over the longer period (24 hours) due to, perhaps, internalisation of peptide. The term internalisation is just speculation because at present nothing is known about it. It is also possible that the effect of RGDS would have been more than observed because the long incubation might have degraded some of the peptide. However it can not be ruled out that all the sites that can bind with peptide bound it at 15μg/ml i.e. the sites are saturated.
The combined result of all the effects outlined above is that RGDS ensures that the adhesion strength never reaches its maximum due to competitive binding.

6.2.2. EFFECT OF YIGSR (tyr-ile-gly-ser-asp) ON THE ADHESION STRENGTH OF L929 AND HELA B CELLS

The control peptide for this experiment was the laminin active site peptide YIGSR which had no effect on adhesion strength at any of the concentration used (figure 6.2 & 6.3). This also gives the interesting findings that since it has no effect, thus it is unlikely that L929 or Hela B cells utilise laminin to any great extent. However the laminin has been shown to increase the strength of L929 and Hela B cell adhesion considerably in serum free medium (chapter 7).

It must be kept in mind that the experiments here, were carried out in serum containing medium. In presence of serum, L929 and Hela B cells might have used fibronectin, vitronectin and vitronectin and collagen respectively instead of laminin. This notion may not be completely true, because laminin may have reinforced the adhesion strength of these cells. If this is to be believed, the YIGSR should depress the adhesion strength of these cells, at least to some measurable extent which it did not. This simple explanation of this failure may be put in the context of rather a large number of proposed recognition sequences reported to date in laminin. That is apart from the YIGSR sequence, at least 8 others have been reported so far (Yamada, 1991). Moreover, in general the peptides derived from laminin appear to have considerably lower affinity of binding to cells than native laminin as suggested by the

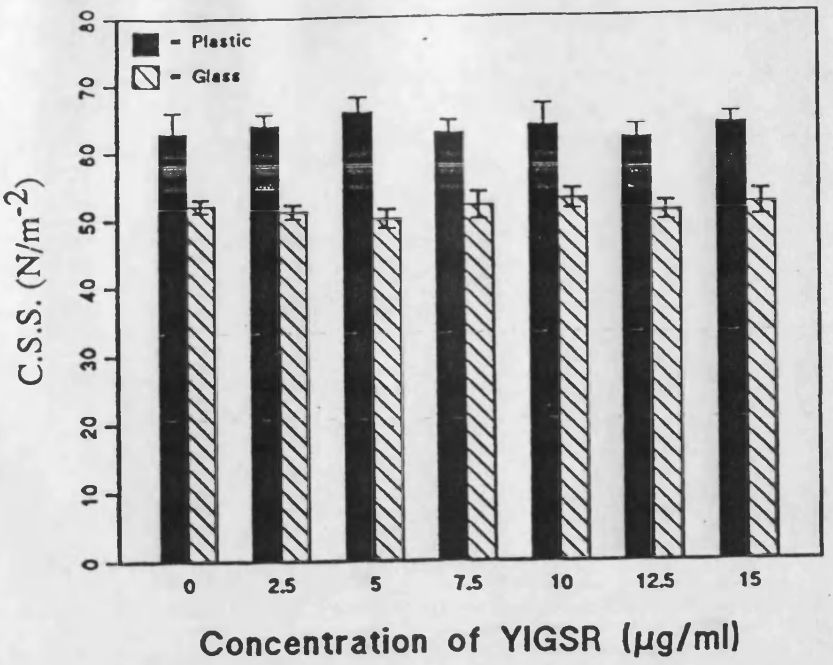


FIGURE 6.2

EFFECT OF YIGSR (tyr-ile-gly-ser-arg) ON THE ADHESION STRENGTH OF L929 CELLS.

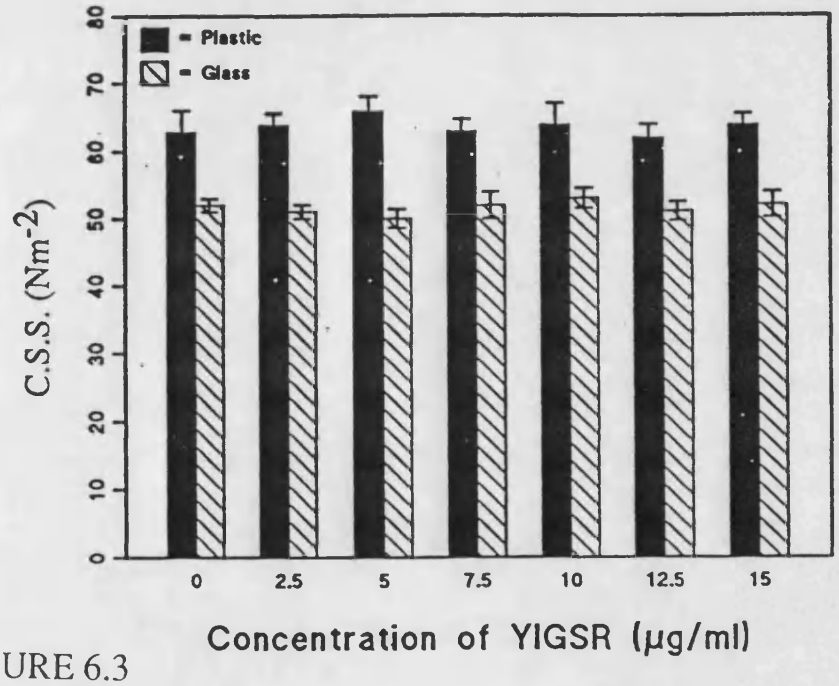


FIGURE 6.3

EFFECT OF YIGSR (tyr-ile-gly-ser-arg-) ON THE ADHESION STRENGTH OF HELA B CELLS.

L929 or Hela B cells were plated on glass or plastic substratum with or without YIGSR peptide at the concentrations indicated on the x-axis of the above two figures (A & B). Adhesion strength of these cells in terms of the critical shear stress (c.s.s.) of detachment was measured according to the standard conditions outlined in materials and methods.

Each data point is representative of 3 experiments. In each experiment, 20 measurements of c.s.s. were made at each concentration of YIGSR. The error bars indicate the standard error of the mean.

A t test indicates the difference in adhesion strength of untreated and YIGSR treated L929 cells is not significant (p=0.45).

higher molar amounts of these peptides compared to laminin for adhesion assays (Sephel et al, 1989). While YIGSR is inefficient in depressing the adhesion strength of both L929 and Hela B cells, of course it might be active in some other cell types. That is each binding site in laminin may be playing a specific role in different cell types.

The findings of the present study are favoured by the work in which it has been shown that a laminin derived peptide RYVVLPR (arg-tyr-val-val-leu-pro-arg-) when coated on the substratum, directly promoted cell adhesion, but when present in solution could not significantly depressed the adhesion (Skubitz et al, 1990). The situation in the present work became very complicated because of the presence of other serum proteins in the culture medium. With these present (fibronectin, vitronectin, collagen 1v) it is unlikely that laminin contributed a measurable part in adhesion of L929 and Hela B cells.

6.3 CONCLUSION

In conclusion, the addition of exogenous RGDS peptide to the serum containing culture medium, perturbed to a significant extent protein-receptor complexes and had a profound effect on the adhesion strength of L929 cells, despite the fact that alternative determinants are present. The main finding of this chapter is that the Microflow chamber appears to be highly sensitive because it can sense a minor change in the environment of the cell adhesion system. This sensitivity is a pre requisite for a competent quantitative technique. It is pleasing that the Microflow chamber not only can detect the changes in the environment of the attached cell but the results obtained with the help of this device can also be used to interpret the biochemical events involved in the cell adhesion. For example RGDS is suspected to block the formation of the protein–receptor bonds. This idea (participation of proteins) was checked by coating the surface with specific adhesion proteins and measuring the adhesion strength of cells growing on this surface with the help of the Microflow chamber as described in the following chapter.

The RGDS might have suppressed adhesion strength by blocking some signals generated by the adhesion proteins. Thus it is suggested in the present and chapter 5 that serum enhances the adhesion strength via adhesion proteins present in it. To check whether adhesion proteins are actually responsible for adhesion strength, surfaces are coated with specific adhesion proteins and adhesion strength measured as described in the following chapter.

CHAPTER 7
THE ROLE OF SUBSTRATUM PRE-ADSORBED PROTEINS IN ADHESION STRENGTHENING PHENOMENA

7.1. INTRODUCTION

In previous chapters, the focus was on the adhesion proteins adsorbed directly from the serum onto the surface. These adsorbed proteins might be encountered by cells in the usual serum supplemented culture medium (Edwards et al, 1987). Attention was also drawn to possible mechanisms by which cells could use one or more protein(s) from serum containing culture medium (chapter 5). Some of these adhesion proteins are now commercially available in their purified form. Taking advantage of their availability, studies were planned to modify the normal tissue culture grade plastic with a specific protein and then find out how this affects cell growth and adhesion. For this purpose the plastic surface of tissue culture was coated with fibronectin or laminin and the effects of this on the adhesion strength of L929 and Hela B cells was examined. Fibronectin and laminin were chosen because it is widely believed that these proteins function with the widest variety of cell types (Couchman et al,1983), with the emphasis that the former has many binding sites on both fibroblasts and epithelial cell surfaces (Dufour et al, 1986). Whereas, laminin binds most favourably with epithelial cells. (Kleinman et al, 1985). The L929 and Hela B cells were selected because the former are fibroblasts and the latter epithelial cells. The results and their interpretations are presented in the following section.

7.2. RESULTS AND DISCUSSION

7.2.1. RESULTS

To evaluate the role of specific protein (fibronectin) in the adhesion strengthening phenomena, tissue culture grade plastic dishes ($55cm^2$) were coated with fibronectin (1-175$\mu g/55cm^2$ dish) overnight as described in materials and

methods. After this time the dishes were washed three times with double distilled water and air dried in a laminar flow for fifteen minutes before seeding the L929 cells on them. At this time four experiments were performed.

1 L929 cells were seeded on fibronectin coated or non coated (control) dishes in the presence of serum in the culture medium and the c.s.s. of detachment was measured after 3 hours.

2 L929 cells were seeded on fibronectin coated or non coated dishes (control) in the absence of serum in the culture medium (Eagles medium plus Nutridoma1%) and the c.s.s. of detachment was measured after 3 hours.

In the next two experiments the cells in NO. 1 & 2 were incubated for 24 hours and c.s.s. of detachment was measured.

When the L929 cells were grown on fibronectin coated dishes in the presence or absence of serum in the culture medium and were observed under phase contrast microscopy, they were found slightly more spread (picture 7.1) compared to the cells growing on non coated plastic in the presence of serum (picture 7.2). The cells grown on non coated dishes in serum free medium were found with round morphology (picture 5.2 in chapter 5).

The results of these experiments are given as below.

1 It was of interest to note that when L929 cells were grown on fibronectin coated dishes in the presence of serum and c.s.s. of detachment was measured after 3 hours, the adhesion strength was increased by 15% over the control value. That is adhesion strength on fibronectin coated surfaces in the presence of serum was $60.0 \pm 1.3 Nm^{-2}$ and on non coated dishes it was $52.1 \pm 1.5 Nm^{-2}$ (figure 7.1).

2 When the L929 cells were grown on fibronectin coated dishes in the absence of serum and c.s.s. of detachment was measured after 3 hours it was increased by 7 fold (700 %) of the control value. That is on the coated dishes in presence of serum free media (Nutridoma 1%) the critical shear was $61.0 \pm 1.4 Nm^{-2}$ and on control dishes it was $7.0 \pm 3 Nm^{-2}$ (figure7.1).

PICTURE 7.1

L929 cells growing on the plastic substratum

PICTURE 7.2

L929 cells growing on the fibronectin pre-adsorbed substratum

3 After 24 hours in the presence of serum on fibronectin coated dishes the c.s.s. was increased by 30% of the control (non coated dishes) value (figure 7.2).

4 After 24 hours in the serum free media on fibronectin coated dishes the adhesion strength was increased by 800% of the control (non coated dishes) value (figure 7.2).

It is clear from the results presented in the figures 7.1 & 7.2 that whether serum is present in the culture medium or not the fibronectin coated dishes enhanced the critical shear value to a maximum of 82.5Nm^{-2}. Furthermore, one important finding is that the dramatic sensitivity of L929 cells in response to a fibronectin coated substratum appears to require a minimum surface density of fibronectin molecules 1$\mu g/55cm^2$ dish, above this level (25-175$\mu g/55cm^2$ dish) the adhesion becomes independent of the added fibronectin concentration.

Now the discussion of these findings follows.

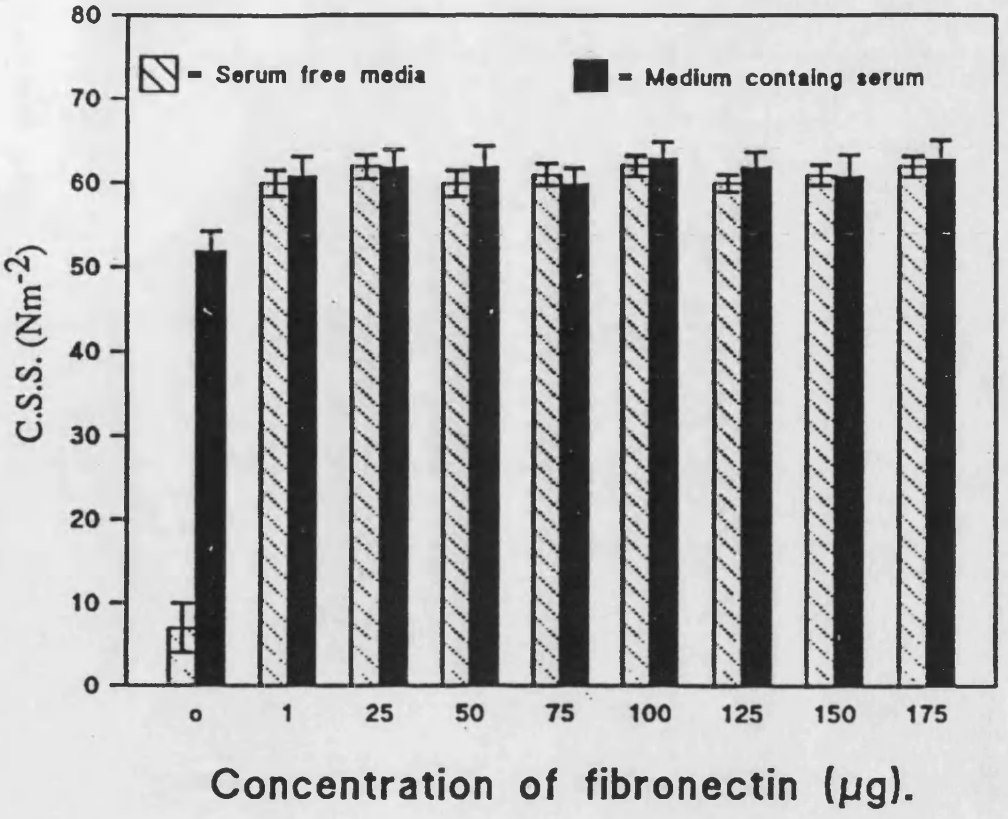


FIGURE 7.1

ADHESION STRENGTH OF L929 CELLS ON THE FIBRONECTIN PRE ADSORBED PLASTIC SURFACE.

The tissue culture grade plastic dishes (55cm^2) were incubated with (0-175 µg/55cm^2) human fibronectin. Fibronectin coating was proceeded as illustrated in materials and methods. L929 cells were grown on fibronectin pre-adsorbed dishes for 3 hours. The adhesion strength of these cells in terms of the critical shear stress (c.s.s.) was measured by using the Microflow chamber. Each data point represents the mean of 30 different determinations, the error bars indicate the standard error of that mean. A t test indicates that the difference between the adhesion strength of cells growing on uncoated dishes (in presence of serum or serum free medium) and fibronectin pre-adsorbed dishes (in presence of serum or serum free medium) is highly significant (p=0.0001).

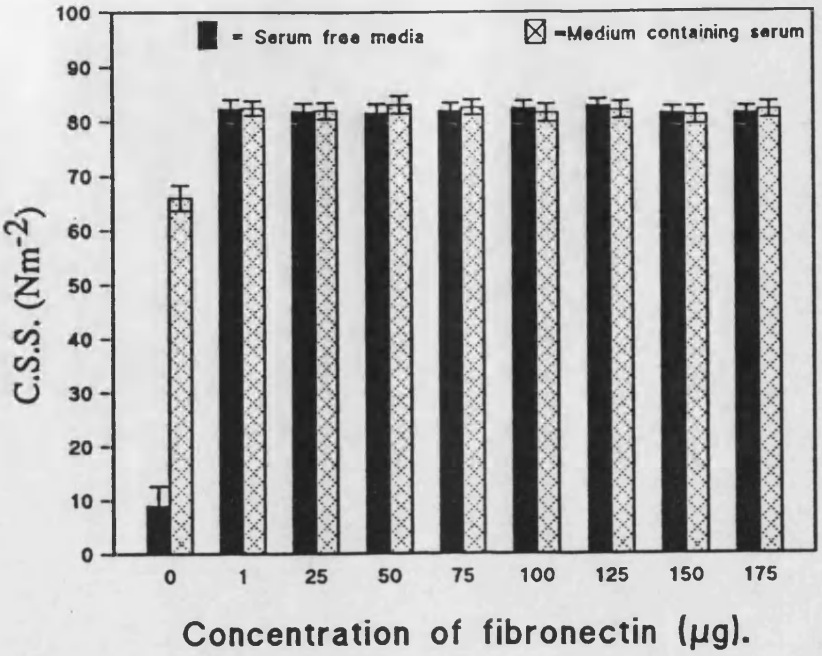


FIGURE 7.2

ADHESION STRENGTH OF L929 CELLS ON THE FIBRONECTIN PRE ADSORBED PLASTIC SURFACE.

The tissue culture grade plastic dishes ($55cm^2$) were incubated with ($0\text{-}175\mu g/55cm^2$) human fibronectin. fibronectin coating was proceeded as illustrated in materials and methods. L929 cells were grown on fibronectin pre-adsorbed dishes for 24 hours. The adhesion strength of these cells in terms of the critical shear stress (c.s.s.) was measured by using the Microflow chamber.
Each data point represents the mean of 30 different determinations, the error bars indicate the standard error of that mean.
A t test indicates that the difference between the adhesion strength of cells growing on uncoated dishes (in presence of serum or serum free media) and fibronectin pre-adsorbed dishes (in presence of serum or serum free medium) is highly significant ($p=0.0001$).

7.2.2 DISCUSSION

The results obtained in response to pre-adsorbed fibronectin were very exciting. Particularly the finding that a very low concentration of fibronectin acts like a catalyst and increases adhesion strength by 9 fold (i.e. $82.5Nm^{-2}$) of the control value (i.e. $9.1Nm^{-2}$). Since fibronectin was simply adsorbed but not chemically coupled to the plastic, the next question was how much of it remained adsorbed on the surface after extensive washing. For this, iodinated fibronectin was used to find out the number of molecules adsorbed per unit area of the tissue culture grade plastic. The details of the procedure are given in materials and methods , however, it is worthwhile to mention that ^{125}I-fibronectin was coated on tissue culture plastic overnight and proceeded exactly the same way as non iodinated fibronectin coating. (see above and materials & methods). The coated ^{125}I-fibronectin was extracted twice with NaOH (1M) and counted on the Gamma counter. The counts per minute and radio specific activity of ^{125}I-fibronectin was used to calculate the number of fibronectin molecules/μm^2.

According to the ^{125}I-fibronectin adsorption experiment, in fact 70% of the added fibronectin was adsorbed to the surface (table 7.1 & 7.2)) For example, when $55cm^2$ ($3x10^{11}\mu m^2$) dishes were coated with 1µg and 150 µg, the number of molecules which remained adsorbed were calculated as 200/μm^2 and 30,000/μm^2 (table 7.1). Interestingly, 200 molecules/μm^2 and 30,000 molecules/μm^2 enhanced the same amount of adhesion strength of L929 cells. Therefore, it was necessary to find out whether 200 molecules/μm^2 are covering the surface underneath the cell or not. For this purpose the following assumption was made.

The fibronectin molecule is a rod shaped molecule with a length of 60-70 nm and a width of 2-3nm (Ito et al,1991). Assuming that fibronectin acquires a flat rectangular shape after its adsorption onto the surface, the area which this molecule will cover can be calculated as $180nm^2$. Therefore, to cover the area of $1\mu m^2$ completely, 5500 molecules are required (see also table 7.2). Thus it is clear that at 1µg/$3x10^{11}\mu m^2$ the fibronectin molecules (200/μm^2) do not completely cover the

Applied FN per $3x10^{11}mm^2$	Molecules/cell	C.S.S. (Nm^{-2}
0 µg	0	9.1 ±3.5
1 µg	2,000	82.5 ±1.5
25 µg	50,000	82 0±1.4
50 µg	100,000	81.7 ±1.5
75 µg	150,000	82 1±1.3
100 µg	200,000	82.5 ±1.2
125 µg	250,000	83 0±1.3
150 µg	300,000	82 2±1.2
175 µg	350,000	81.5 ±1.2

TABLE 7.1

^{125}I-fibronectin was coated on the tissue culture grade plastic as illustrated in materials and methods. The number of adsorbed fibronectin (FN) molecules/cell ($10\mu m^2$) was calculated as described in materials and methods. The critical shear stress of detachment (c.s.s.) of L929 cells growing on this adsorbed fibronectin was measured in terms of Nm^{-2}. Further details may be found in the text and in the figures 7.1 & 7.2.

L (nm)	W (nm)	A (nm^2)	Molecules required/cell
60	3	180	55,000

Table 7.2

On the basis of theoretical estimated size of adsorbed fibronectin, number of molecules required to cover the underneath of the cell surface was calculated by assuming the size of cell $10\mu m^2$. Further details may be found in the text.

whole surface. As indicated above and making the assumption on the size of the surface bound fibronectin molecule, there are $5x10^4$ molecules needed per cell (assuming that the area of L929 cell is $10\mu m^2$). However, the maximum cell adhesion is found at $2x10^3$ molecules per cell, a value 30 times lower than expected. Moreover, the L929 cells gained maximal adhesion ($82.5\pm 1.5Nm^{-2}$) on these minimal fibronectin molecules. That is, $2x10^3$ fibronectin molecules caused the cell adhesion to increase from $9.1 \pm 3.5\ Nm^{-2}$ to $82.5 \pm 1.5Nm^{-2}$, a nine fold increase.

Intuitively the addition of 2000 fibronectin molecules to a cell could not increase the critical shear stress by the factor of nine seen in figure 7.1. Some workers have tried to develop mathematical models which describe adhesion of a cell to a substratum. One of these (Bell, 1978) calculates that an "average" receptor-ligand bond, such as that which occurs between a lectin and its target protein, has a strength of $0.4x10^{-5}$ dynes. An immediate problem is that this figure is valid only for cells which are pulled in a perpendicular manner, away from their substratum. In the hydrodynamic Microflow chamber the liquid force applied to the cell is parallel to the cell's substratum. In other words the cell can be likened to a small submarine anchored to the bottom of a fast flowing river (the Microflow chamber). The moving water exerts a force on the leading edge or front of the submarine with little or no force exerted along its length including its trailing edge. The same applies to a cell in the Microflow chamber where the leading edge of the cell takes the whole force. Imagine now an idealised rectangular cell with regularly spaced ligand-receptor binding this cell to the substratum (figure 3.5). At one time the only important binding sites are at the leading edge. Once sufficient force has been applied to peel these bonds away from the substratum, all the force is taken on the second row, then the third row and so on while the cell rolls off the substratum. It is not possible, therefore, to calculate the actual quantitative force to disrupt the added 2000 fibronectin molecules. One can however make an educated or qualitative estimate.

It is probably reasonable to suggest that at most, 10% of the receptor-ligand sites are at the cells leading edge,that is, 200 molecules of fibronectin. Intuitively it seems improbable that these 200 molecules can increase the overall adhesion of the cells by a factor of 9 or so (figure 7.1). This being the case, it is reasonable to suggest a strong possibility that the fibronectin molecules are acting as a signalling system. This possibilityis described in detail below.

7.2.2.1. SIGNALLING MECHANISM

As stated above it seems likely that there must be some signalling mechanism involved in the fibronectin enhanced adhesion strength. At present nothing is known about this signalling mechanism, therefore, the topics discussed below are speculations. It is not out of place to mention that the object of this work is not to explore the signalling mechanism, thereby, the emphasis on these findings must be that the Microflow chamber (developed in present work) is a device which is able to determine the effect of 200 molecules/μm^2. It is pleasing that this device can be used at the molecular level. This statement no way detracts from the importance of the findings of the fibronectin enhanced adhesion strength. Thus coming back to the signalling mechanism the following possible speculations concerning this mechanism can be considered.

1 Involvement of the adhesion bonds.

2 Adhesion strengthening via formation of the adhesion plaques and the cytoskeletal orgnisation.

(a) cAMP

(b) Phosphorylation

(c) Proteolysis

3 The distance between cell and the surface

7.2.2.1.1. INVOLVEMENT OF THE ADHESION BONDS

A theory of an adhesion signalling system was put forward in the chapter 4. The signal might be triggered by the fibronectin-integrin bonds and acts as a stimulator of biochemical and cytoskeletal events involved in gaining the adhesion strength. It is likely that a certain number of initial bonds are required to initiate this process. These specific bonds somehow send a signal to the interior of the cell machinery to recruit the cytoskeletal proteins to produce more stable and compact adhesion.. It is also possible that this triggering low level of fibronectin (2000 molecules/cell) stimulates the synthesis and secretion of endogenous fibronectin or other protein(s) which later bind with their respective receptors thus contributing to the final adhesion strength. These speculations may be oversimplifying the situation. It could be that a variety of stimuli are involved by which cell adhesion is strengthened. Some of these possible stimuli are discussed as below.

7.2.2.1.2. ADHESION STRENGTHENING VIA FORMATION OF THE ADHESION PLAQUES AND THE CYTOSKELETAL ORGNISATION

Once the initial bonds have formed, these bonds must be triggering the biochemical events responsible for the formation of the adhesion plaques (see chapter 1) and the cytoskeletal orgnisation (the coupling of the cytoskeletal proteins with each other and with integrins, see chapter 1)). At present these biochemical events are not known. However, some of the possible mechanisms are discussed below.

(a) cAMP

cAMP could be suspected to play a role as an adhesion signalling system in the activation of adhesion phenomena. This molecule may be affecting this process through cAMP dependent protein kinases. It has been known for some time that cell adhesion can be strengthened by raising the intracellular level of cAMP (Johnson & Pastan, 1972). Although qualitatively, they were able to show that the detachment of the cells was retarded by artificially raising cAMP inside the cell. Still qualitative,

many studies showed that virally transformed cells responded to elevated cAMP by adhering more tightly to their substratum (Leader et al, 1983).

Nevertheless if the cAMP is involved it must be acting through the protein kinases. Here the possibility is that the binding of fibronectin with its receptor may induce conformational changes in the cytoplasmic portion of the fibronectin receptors which may lead the events which elevate the cAMP level in the cell and in turn the protein kinases may be activated. The protein kinases may be stabilizing the adhesion protein-receptor-cytoskeleton complex via the phosphorylation of some of the participant in this complex as stated below.

(b) PHOSPHORYLATION

The protein kinases can phosphorylate the cytoplasmic portion of the receptor or some of the cytoskeletal proteins. In fact phosphorylation appeared to stabilize integrin-cytoskeleton interactions (Suzuki et al, 1987). This stabilization in turn may stabilize the fibronectin-integrin complex and thereby contribute to the formation of the stable adhesion plaques. These adhesion plaques may be an important factor in gaining the final adhesion strength of a particular cell line (e.g. L929 cells). Apart from phosphorylation, proteolysis could be another aspect of regulation of cell adhesion strength which I propose is under the control of fibronectin activation system as is illustrated below.

(c) PROTEOLYSIS

Werb et al (1989) found that the blockage of fibronectin receptor by using monoclonal antibodies actually enhanced the expression of two extracellular degrading enzymes, collagenase and stromolysin. It is possible that in my work, apart from sending a signal for cytoskeletal orgnisation and thus adhesion plaque formation, fibronectin suppressed the expression of proteolytic enzymes and thereby increasing the adhesion strength (by protecting the extracellular proteins).

7.2.2.1.3. THE DISTANCE BETWEEN THE CELL AND THE SURFACE

It is generally believed that focal adhesions are sites, the presence of which may be correlated with increased adhesion strength (Norton & Izzard, 1982). This suggestion was made on the basis of qualitative observations. However, In the presence of fibronectin precoating, the average distance of 20nm between the cell and the substratum was classified as an adhesion plaque. Whereas, in the case of an uncoated substratum, but in the presence of serum, a distance of 50nm was defined as an adhesion plaque (Schakenraad et al, 1989). Thus it can be implied that, the smaller the distance the stronger the adhesion. Therefore, in the present experiments involving the surface precoating with fibronectin, the critical step might be the determination of adhesion strength via decreasing the distance between the cell and its substratum. This decreasing distance system must be under the control of the fibronectin activation system.

7.2.3 EFFECT OF PREADSORBED FIBRONECTIN ON THE ADHESION STRENGTH OF L929 CELLS IN PRESENCE OF SERUM

Another important finding in this work was that once the surface is coated with fibronectin, the presence of serum does not play any further role in strengthening the adhesion. Here the situation is puzzling concerning the proposed fibronectin removal from adhesion plaques. This is because Grinnell (1986) found that adsorbed fibronectin could only be removed from beneath the focal adhesion in the presence of serum. Except that Grinnell speculated mechanical desorption of fibronectin, no other explanation about this finding was given In my study the fibronectin was coated onto the surface and L929 cells were seeded in the culture medium containing serum. Interestingly the adhesion strength was not perturbed and no difference in the c.s.s. of detachment from fibronectin substratum was noted in the presence or absence of serum.

In the present work the desorption of fibronectin underneath the cell was not checked, since it was not the main object of this study. However, the results obtained

in the presence of serum indicate that once fibronectin activated initial events occur and the sequence of events leading to final adhesion strength is started, then adhesion strength is independent of the presence of serum. In context of the serum experiments, one could argue that on non coated plastic, fibronectin which is present in the serum does not increase as much adhesion as was observed in response to the pre-adsorbed fibronectin. This argument further strengthens due to the fact that about 38ng/cm^2 fibronectin adsorbed on the plastic when it is incubated with the medium containing 10% foetal calf serum (Steele, et al, 1991). This amount is double the amount of pre-adsorbed fibronectin in the present study. That is in present study 18ng/cm^2 was adsorbed when 1µg was used for adsorption on the 55cm^2 dish.

The simple answer might be that fibronectin adsorbed from the serum is not as biologically suitable as was the precoated fibronectin substratum. Serum is a complex mixture of proteins and other molecules. 38ng fibronectin/cm^2 is such a small amount compared to other molecules that other proteins do not allow fibronectin to alter its conformation to a suitable form for full activation of adhesion strength. This idea gains support from the other lines of evidence which suggest that not only is the quantity of protein adsorbed on the surface important but also their conformation is a determinant for cell adhesion (Fabrizius- Homan & Cooper,1991).

After establishing the fact that fibronectin acts as an activator rather than mediator in cell adhesion, it was desirable to know whether other adhesion proteins play similar roles in this complex process. It was with this question in mind that laminin was selected so that its role in strengthening the cell adhesion could be examined.

7.2.4 EFFECT OF PRE-ADSORBED LAMININ ON THE ADHESION STRENGTH OF L929 AND HELA B CELLS

Laminin was obtained from Sigma and coating of plastic petri dishes with this protein was proceeded with as stated in the materials and methods. Briefly, 1-175µg laminin was dissolved in PBS and the 55cm^2 dishes were coated overnight. Thereafter, immediately before use, unadsorbed laminin was extensively removed

with PBS. It is not known how much laminin was adsorbed on the surface. For this purpose laminin needed to be iodinated and the adsorption determined as illustrated in case of fibronectin. However, financial considerations ruled this out. Nevertheless on laminin coated dishes the following experiments were performed.

1 L929 and Hela B cells were seeded on laminin coated (1-175μg/ 55cm^2 dishes) or non coated (control) dishes in the absence of serum and c.s.s. of detachment was measured after 24 hours (see materials and methods).

2 L929 and Hela B cells were seeded on laminin coated (1-175μg/55cm^2 dishes) or non coated (control) dishes in the presence of serum (55cm^2) and c.s.s. of detachment was measured after 24 hours (see materials and methods)

The following results were obtained. It was of interest to note that like fibronectin, laminin too, at lower or higher concentration increased adhesion strength substantially. For example, the laminin coated substratum enhanced the c.s.s. of detachment of L929 cells 7 fold (65.0 ±1 Nm^{-2}) of the control value (9.1±3.5 Nm^{-2}) whereas the increase in Hela B cells was 2.5 fold (63.1 ±2.3Nm^{-2}) of the control value 23.0±6Nm^{-2} (figure7.3)

Unlike fibronectin, laminin did not increase adhesion strength of L929 and Hela B cells in the presence of serum. That is, in the presence of serum the adhesion strength of these cells on laminin coated (e.g. 65.0± 1.4 Nm^{-2} for Hela B and 64.1 ± 1.2Nm^{-2} for L929 cells) and non coated plastic dishes (e.g. 66.0 ±2.5 Nm^{-2} for Hela B and 66.1±1.6Nm^{-2} for L929 cells) was the same. In contrast fibronectin activation caused a 30% increase in the c.s.s. of detachment of L929 cells compared to an uncoated substratum in the presence of serum (figure 7.3).

In numerous studies, laminin is considered best for epithelial cells (Klein et al, 1988). It was expected that laminin would enhance adhesion strength favourably for Hela B cells (epithelial cell line) compared to L929 cells (fibroblasts). Surprisingly the adhesion strength of L929 cells (65.0Nm^{-2}) was more enhanced compared to Hela B cells (63.1Nm^{-2}). Thus the observations that led to these findings were unexpected.

However, the main difference was found to be between fibronectin and laminin activation of the adhesion phenomena.

At present no specific reason concerning the different behaviour of L929 cells toward these proteins is available. However, the number of receptors on the L929 cells which might bind selectively to fibronectin and laminin may account for the difference in the enhancement of adhesion strength caused by these proteins.

As stated earlier, from the results presented in figure (7.3) it is noted that laminin, like fibronectin (though not equivalent to fibronectin) at low concentrations ($1\mu g/55cm^2$ dish) substantially increased the adhesion strength of L929 cells (7 fold). It implies that at lower concentrations of laminin a similar phenomena is operative as was seen for fibronectin . Therefore, it is likely that laminin too, is signalling to the interior of cells for activating the events leading to the final adhesion strength of cells. According to this speculation a small number of laminin molecules might have initiated the signalling for intracellular development of the full adhesion strength, via a signalling system. Thus the discussion about fibronectin may also be valid for laminin.

7.3 CONCLUSION

The results imply that fibronectin and laminin participate in adhesion as activator molecules rather than only attachment factors. It is seen in the figures7.1 to 7.3 that L929 and Hela B cells possess a specific critical shear value with regard of uncoated plastic, fibronectin coated plastic and laminin coated plastic. Therefore, the major finding of this chapter is that the c.s.s. of detachment is not only specific for specific cell line but also is specific for substrates.

A specific value in response to pre- adsorbed specific proteins can be implemented in a variety of biomedical and biotechnological processes and thus may be a breakthrough in mammalian cell technology, where rather than guessing, a specific critical shear value can be used confidently before proceeding with any process. As revealed in the discussion, the feeling that adhesion strengthening

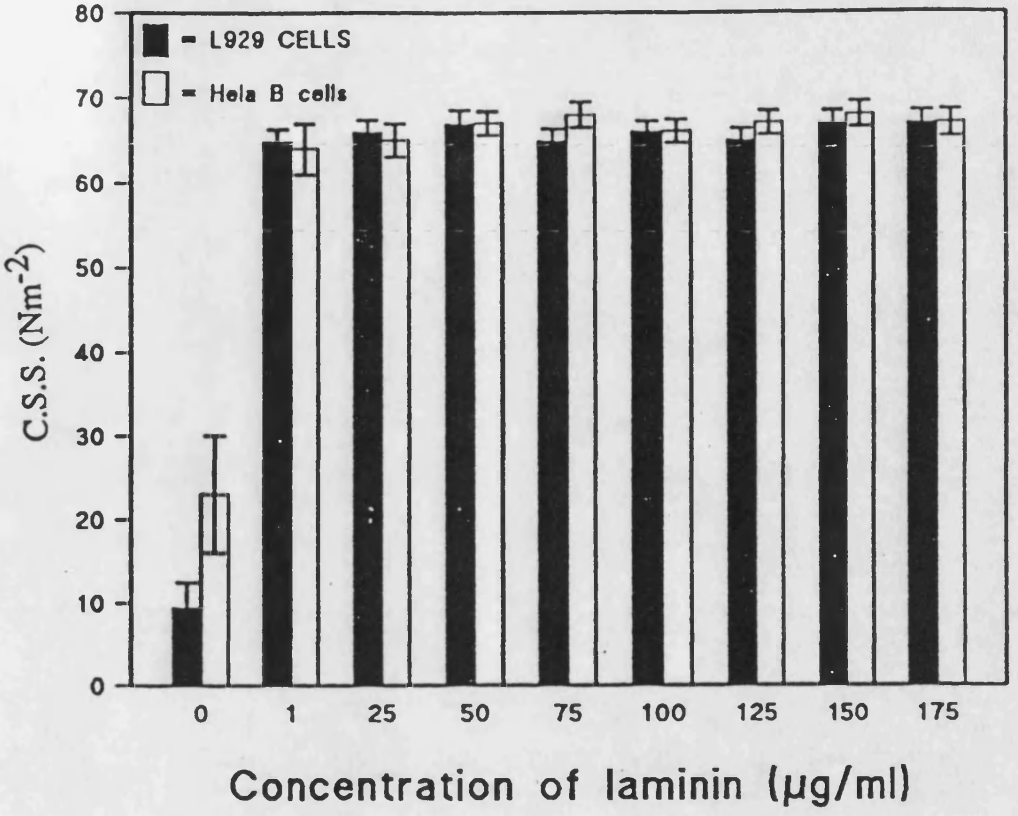


FIGURE 7.3.

ADHESION STRENGTH OF L929 AND HELA B CELLS ON THE LAMININ PRE-ADSORBED PLASTIC SURFACE IN THE ABSENCE OF SERUM

The tissue culture grade plastic dishes (55cm^2) were incubated with (0-175µg/55cm^2) Englebreth Holm-Swarm mouse sarcoma laminin. Laminin coating was proceeded as illustrated in materials and methods. L929 or Hela B cells were grown on laminin pre-adsorbed dishes for 24 hours. The adhesion strength of these cells in terms of the critical shear stress (c.s.s.) of detachment was measured by using the Microflow chamber.

Each data point represents the mean of 20 different determinations. The error bars indicate the standard error of that mean. A t test indicates that the difference between the adhesion strength of both cell lines growing on uncoated or laminin coated dishes is highly significant (p=0.0001).

phenomena is under the control of signalling mechanisms became more strong when only a few molecules were found to be enhancers of the maximum adhesion strength. However, as stated earlier, it must be emphasized that the aim of this work was not to dissect the second messenger system, if indeed it exists. Intuition suggests that there is a way at least to implicate a second messenger. The approach taken was, to see if cell adhesion and adhesion strengthening could be linked to protein synthesis. The detailed arguments will be given later (results and discussion of the following chapter). In start, if protein synthesis affects cell adhesion and its strengthening then they must be linked. This link must be a feedback mechanism whereby the level of protein synthesis (of cell adhesion proteins) is tied to the adhesion state of the cell. That is protein synthesis is up and down regulated as adhesion proteins are required or not.

The protein synthesis experiments are described in the next chapter.

CHAPTER 8

ROLE OF ENDOGENOUS PROTEIN(S) IN STRENGTHENING THE ADHESION OF L929 CELLS.

8.1 INTRODUCTION

In the previous chapters it has been proposed that the first phase is receptor driven which leads to the activation of my proposed second phase via an unknown control system. While for the first phase, the serum used in the culture medium may provide most of the adhesion proteins initially deposited on the substratum, the cells themselves are responsible for synthesizing extracellular adhesion protein(s). (Grinnell & Feld, 1980). The endogenously synthesized matrix components are secreted by the cells and incorporated into the developing matrix. This process might lead them into second phase. The cells that produce the extracellular matrix protein(s) also express surface receptors for these components to make adhesion bonds. Recent studies have shown that the continued adhesion of cells to the substratum is dependent on endogenously produced matrix molecules (Couchman et al, 1983). Therefore cell-extracellular matrix interactions change continuously in the expression of the extracellular matrix protein(s) and their receptor(s).

Despite these findings, a direct relationship between expression of cell adhesion proteins and cell adhesion has been difficult to establish due to the lack of quantitative studies. That is to say, cells in the presence of serum or purified adhesion protein(s) will attach and probably spread normally even in the absence of protein synthesis (Neumeir & Reutter, 1985), However, qualitative studies can not detect any difference between cell adhesion in the presence or absence of protein synthesis. Once again, credit goes to the Microflow chamber, developed in this present work. That is, it is possible to distinguish between the contribution of exogenous and endogenous protein(s) to cell adhesion.

For this purpose, by using Microflow chamber, the possible role of on going protein synthesis which is controlled by a second messenger in cell adhesion

strengthening can be established. To achieve these goals in the present chapter, metabolic inhibition was employed to evaluate the participation of endogenous proteins in the cell adhesion process. Inhibition of protein synthesis was accomplished by treating L929 cells with emetine and cycloheximide while the secretion of some adhesion protein (s) (fibronectin) was perturbed by employing the sodium ionophore, monensin (materials & methods). The results presented in the following pages suggest the beginning of an enormous breakthrough in establishing the mechanism of strengthening adhesion by an adhesion protein activated second messenger system. The results and discussion of these studies now follows.

8.2 RESULTS & DISCUSSION.

8.2.1. DETERMINATION OF EFFECTIVE DOSE OF EMETINE OR CYCLOHEXIMIDE FOR PROTEIN SYNTHESIS INHIBITION

To determine the concentration of emetine and cycloheximide which effectively arrests the growth of cells, a family of curves has been obtained (figures 8.1 & 8.2). It was determined that although 0.1μg/ml and 0.5μg/ml of cycloheximide and emetine substantially inhibits the growth of L929 cells, it is 1μg/ml of these drugs which almost completely stopped the growth of the L929 cells (figures 8.1 & 8.2). When L929 cells were labelled with [^{35}S]-metionne, inhibition of protein synthesis in response to emetine and cycloheximide was noted to be 97.5% and 95% respectively. Surprisingly it was found that even after 8 hours, 3% and 5% protein synthesis was still going on in response to 1μg/ml of emetine and cycloheximide respectively (figures 8.3 & 8.4). Coupling of growth experiments with this biosynthetic labelling study showed that 1μg/ml of these drugs is an appropriate concentration to work with. To look at the effects of protein synthesis inhibition bythese drugs on the adhesion strength of L929 cells, the following approaches were made.

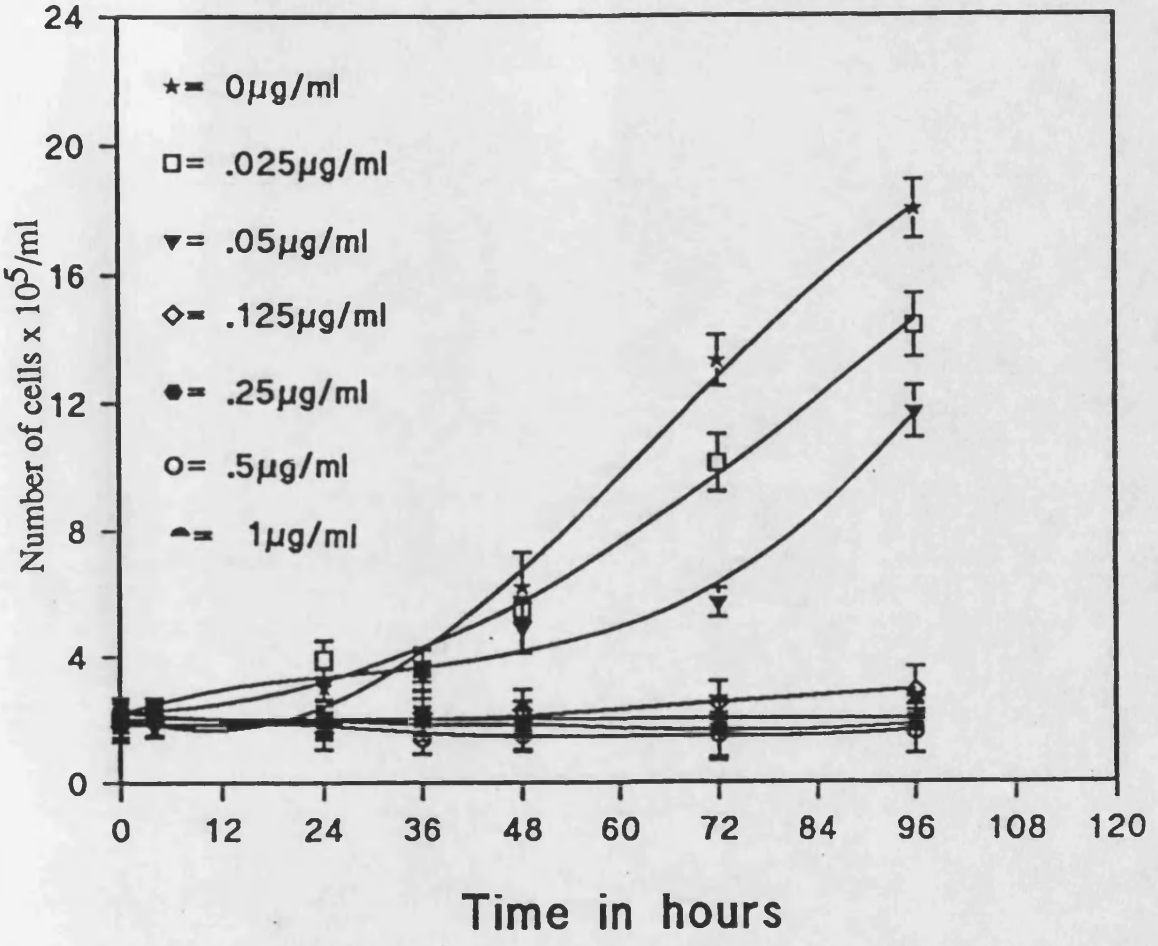


FIGURE 8.1

EFFECT OF CYCLOHEXIMIDE ON THE GROWTH OF L929 CELLS..

Sub confluent L929 cells were trypsinised and inoculated in the tissue culture flasks (25 cm^2). The dose of cycloheximide which effectively inhibited growth of these cells was determined by adding different concentrations of this drug (indicated in the frame of figure) at the beginning of the experiments which lasted for 96 hours. Each data point represents 6 experiments in each of which cells were counted for 10 times. The error bars indicate the standard error of the mean. Where error bar does not appear, it is smaller than the symbol. Further details of the procedure may be found in the text.

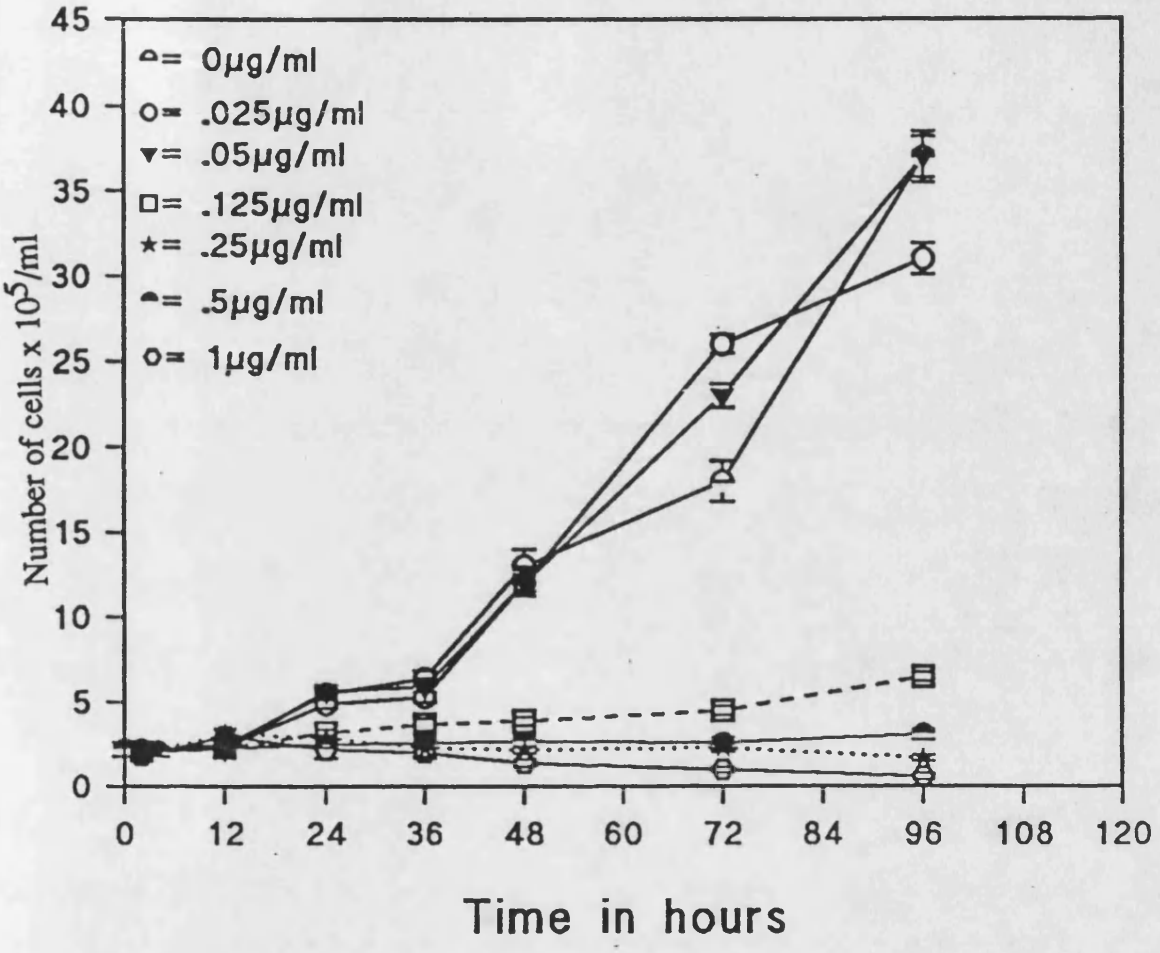


FIGURE 8.2

EFFECT OF EMETINE ON THE GROWTH OF L929 CELLS.

Sub confluent L929 cells were trypsinised and inoculated in tissue culture flasks. The dose of emetine which effectively inhibits growth of these cells was determined by adding different concentrations of this drug(indicated in the frame of figure) at the beginning of the experiment which lasted for 96 hours. Each data point represents 6 experiments in each of which cells were counted for 10 times. The error bars indicate the standard error of the mean. Where error bar does not appear, it is smaller than symbol. Further details of the procedure may be found in the text.

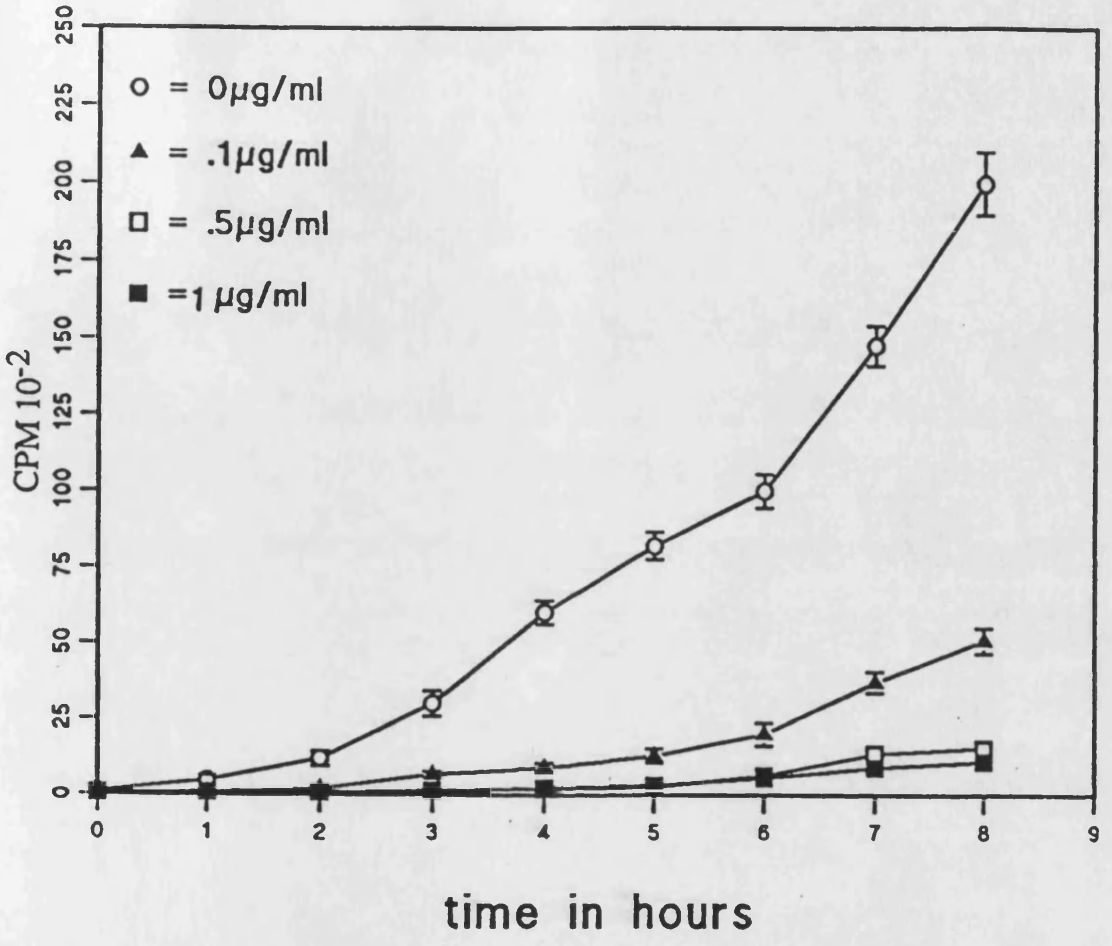


FIGURE 8.3

PROTEIN SYNTHESIS IN L929 CELLS IN RESPONSE TO CYCLOHEXIMIDE.

Adherent cultured L929 cells were plated at a density of $5x10^4$ cells/cm^2 in the presence or absence of cycloheximide (concentrations are indicated in the frame of this figure) and allowed to attach to the 24 well plate for 2 hours. At this stage these cells were metabolically labelled with [^{35}S]-methionine. The incorporation was followed over a period of 8 hours. At time ranging from 0 to 8 hours, the cells were sequentially prepared for scintillation counting as illustrated in materials and methods. The results are presented in terms of cpm/hour/$1x10^5$ cells.

Each data point represents 5 different experiments in each of which the effect of different concentrations of cycloheximide was examined in triplicate. The error bars indicate the standard error of the mean. Where error bars are not apparent, they are smaller than the symbols. Further details may be found in the text.

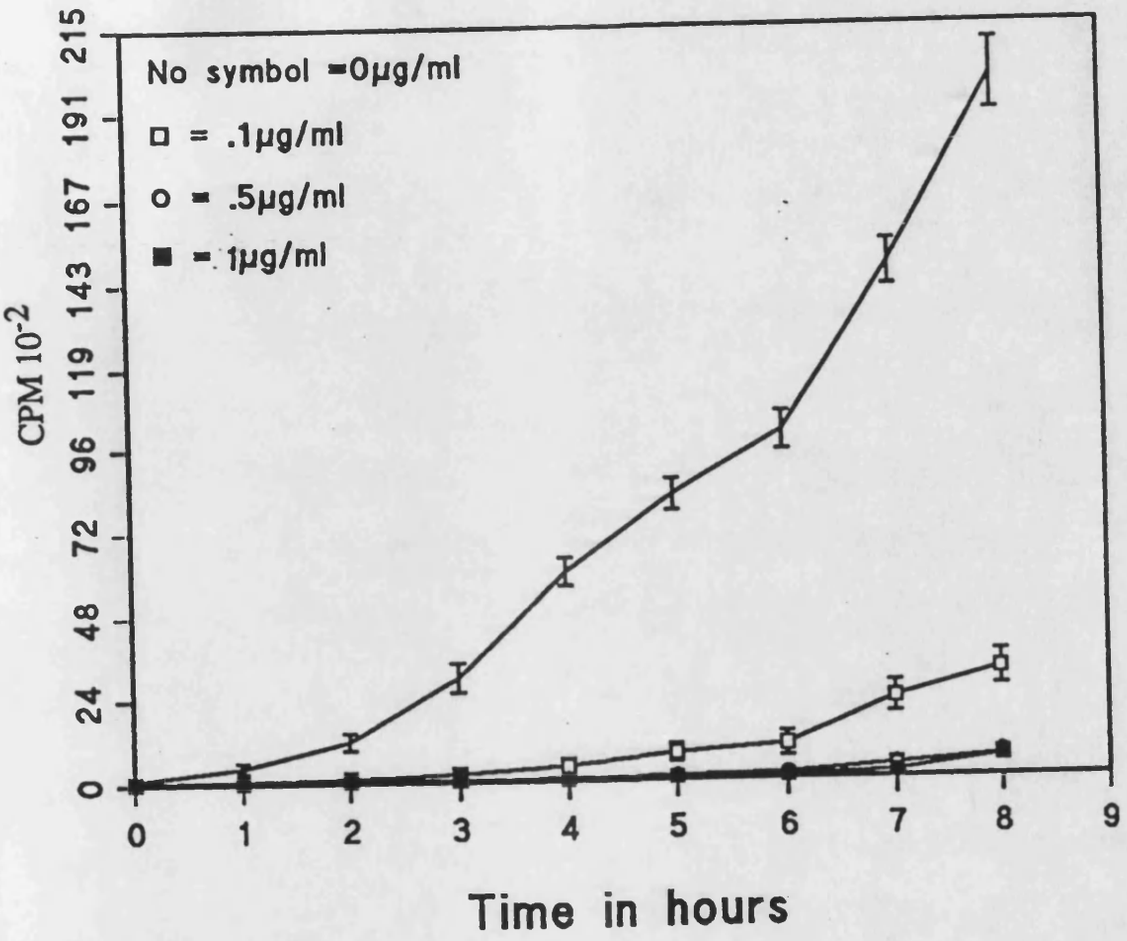


FIGURE 8.4

PROTEIN SYNTHESIS IN L929 CELLS IN RESPONSE TO EMETINE.

Adherent cultured L929 cells were plated at a density of $5x10^4$ cells/cm^2 in the presence or absence of emetine (concentrations are indicated in the frame of this figure) and allowed to attach to the 24 well plate for two hours. At this stage these cells were metabolically labelled with [35_S] methionine. The incorporation was followed over a period of 8 hours. At times ranging from 0 to 8 hours, the cells were sequentially prepared for scintillation counting as illustrated in materials and methods. The results are presented in terms of cpm/ hour/$1x10^5$ cells.

Each data point represents 5 different experiments in each of which the effect of different concentrations of emetine was examined in triplicate. The error bars indicate the standard error of the mean. Where error bars are not apparent, they are smaller than the symbol. Further details may be found in the text.

8.2.2. EFFECT OF EMETINE, CYCLOHEXIMIDE OR MONENSIN ON THE ADHESION STRENGTH OF SUB-CONFLUENT L929 CELLS

In the initial experiments, 1μg of emetine or cycloheximide per ml of medium were employed to inhibit protein synthesis in the L929 cells which had already been growing for 24 hours. This was carried out by replacing the old medium (containing serum or serum free) with or without drug and further incubating the cells with these drugs for 3 or 6 hours. After this time, the critical shear stress of detachment was measured. There were no observable effect of these drugs on the critical shear values, either in the presence or absence of serum in the medium. That is the control (untreated) and the drug treated L929 cells showed the same detachment values for the adhesion strength (figure 8.5).

One explanation of this finding is that the cells were carrying on traces of protein synthesis (3-5%), even after substantial protein synthesis inhibition (figures 8.3 & 8.4). This protein synthesis at lower level (3-5%) might not be sufficient for cell division as the number of cells did not increase in presence of 1μg/ml of these drugs. However, even 3-5% protein synthesis was found to be supportive for the adhesiveness of L929 cells. The synthesis of which protein (adhesive or others) was inhibited remains to be determined It might be the part of the cells internally retained pool of adhesion proteins, is they secreted later, the end result being no change in adhesion strength. The insensitivity of L929 cells after 3-6 hours of drug treatment (figure 8.5) indicates that the binding sites of the proteins involved in the adhesion process may have a long half life and therefore, were not affected by the relatively brief drug treatment.

Alternatively, before protein synthesis inhibition occurred, the adhesion proteins from the serum plus the cell secreted proteins might have sent a signal to the cell machinery to acquire its final adhesion strength. Once the signals have been

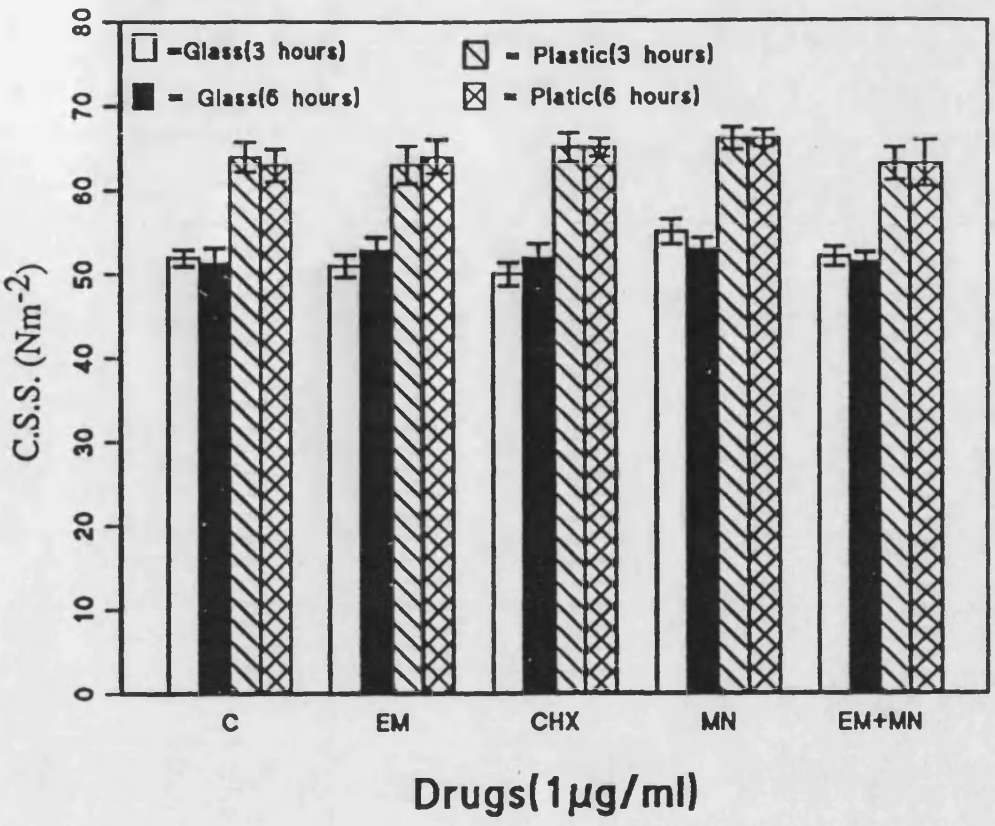


FIGURE 8.5

ADHESION STRENGTH OF SUB CONFLUENT L929 CELLS TREATED WITH EMETINE (EM) OR CYCLOHEXIMIDE (CHX) OR MONENSIN (MN) OR EMETINE PLUS MONENSIN (EM+MN) FOR 3 OR 6 HOURS.

The old culture medium of sub confluent L929 cells was replaced with fresh medium containing 1µg/ml of emetine or cycloheximide or monensin or emetine plus monensin. The treated (EM, CHX, MN, EM+MN) or untreated (C) cells were further incubated for 3 or 6 hours. After this time adhesion strength was measured and the results are presented in terms of the critical shear stress (c.s.s.) of detachment. A t test indicates insignificant difference($p=0.53$) in the adhesion strength of treated or untreated cells.

transduced by these adhesion proteins and are transmitted to inside the cell, then no matter whether protein synthesis is going on or not, the cells will strengthen their adhesion in the presence of exogenously supplied (serum) adhesion proteins. This argument in no way detracts from the fact that cell adhesion is an active phenomenon. Some workers have reported observations that can be interpreted as supporting to the above notion. For example Grinnell & Feld (1979) found a small pool of adhesion proteins inside the cells even after protein synthesis inhibition. Taken together these observations and findings of this experiment (where cells were treated for 3-6 hours) it can be concluded that simply stopping protein synthesis at the time of the adhesion experiments may not be sufficient. That is the small internal pool of adhesion proteins retained by the cells most likely will serve as a source of proteins needed in the rest of the events involved in strengthening the cell adhesion.

To abolish this possibility, the next experiment was designed to examine the combined effects of emetine and monensin on the adhesion strength of L929 cells. The monensin was used because of its reported effects on the secretion of some adhesion protein(s) from the cells (Uchida et al. 1979). However even in these experiments, the critical shear values remained unaltered in both the presence or absence of serum in the culture medium (figure 8.5). It is not out of place to mention that the morphology of the cells was not changed in either of the experiments described above. One could argue that monensin should stop secretion of adhesion protein(s) from this internal pool and thereby prevent participation of adhesion protein (s) in cell adhesion. This possibility could be ruled out on the basis that among the known adhesion proteins, it is only fibronectin, secretion, which is impeded. The fate of other adhesion molecules in response to monensin has yet to be determined. In addition, this short term treatment with monensin might not be sufficient to completely block the secretion of fibronectin. In fact, contrary to other reports (Mollenhauer et al, 1990) the overall secretion was enhanced in the presence of monensin used in the present study (figure 8.6). This enhanced secretion was not simply due to the reason that monensin made the L929 cells leaky, since the cells did

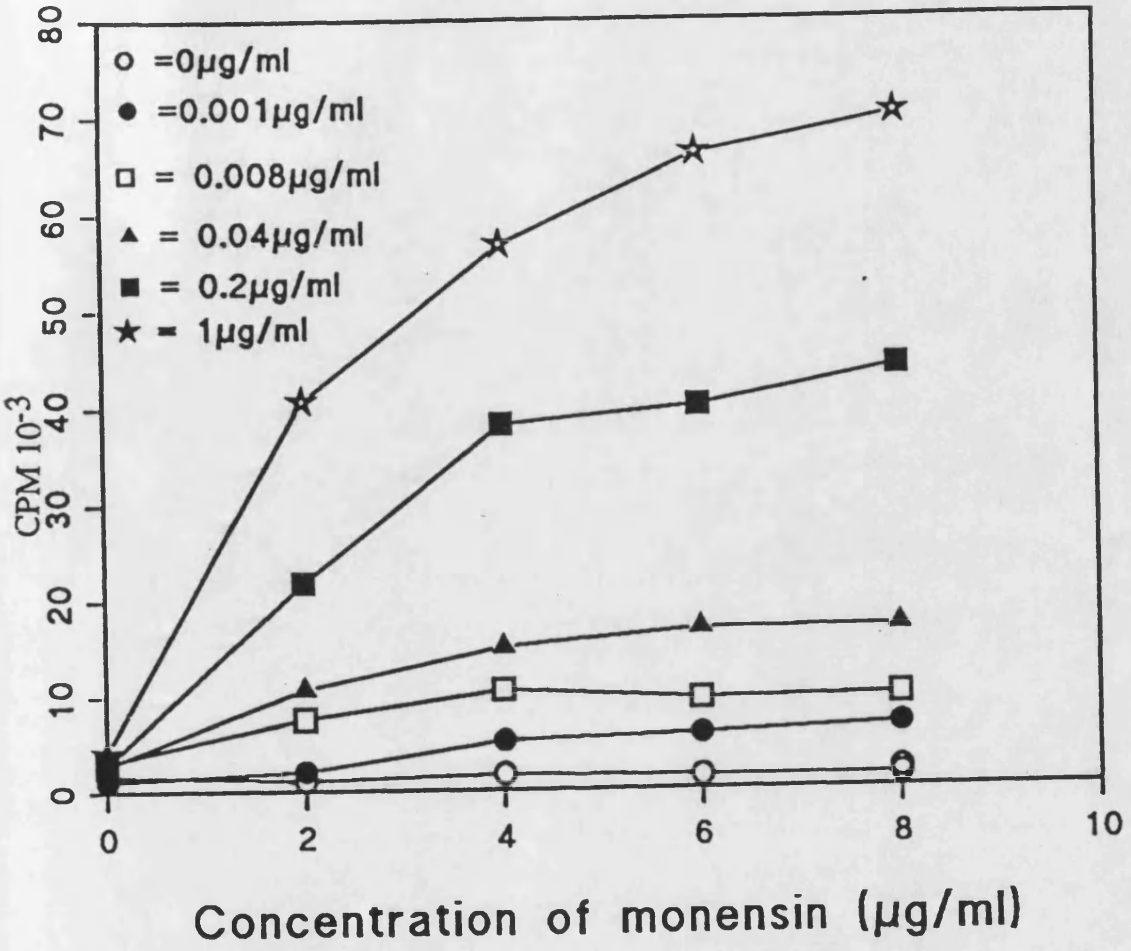


FIGURE 8.6

MONENSIN ENHANCED SECRETION OF ACID INSOLUBLE MATERIAL FROM THE L929 CELLS.

L929 cells were metabolically labelled with [35_S] methionine far overnight. The labelled medium was replaced with the fresh serum free media (with or without different concentrations of monensin) as described in materials and methods. The conditioned medium was collected at different time intervals (indicated on the x-axis of the figure) and proceeded for the scintillation counting as illustrated in chapter 2.

The results are expressed as counts per minute per $1x10^6$ cells/2hours. Further details may be found in the text.

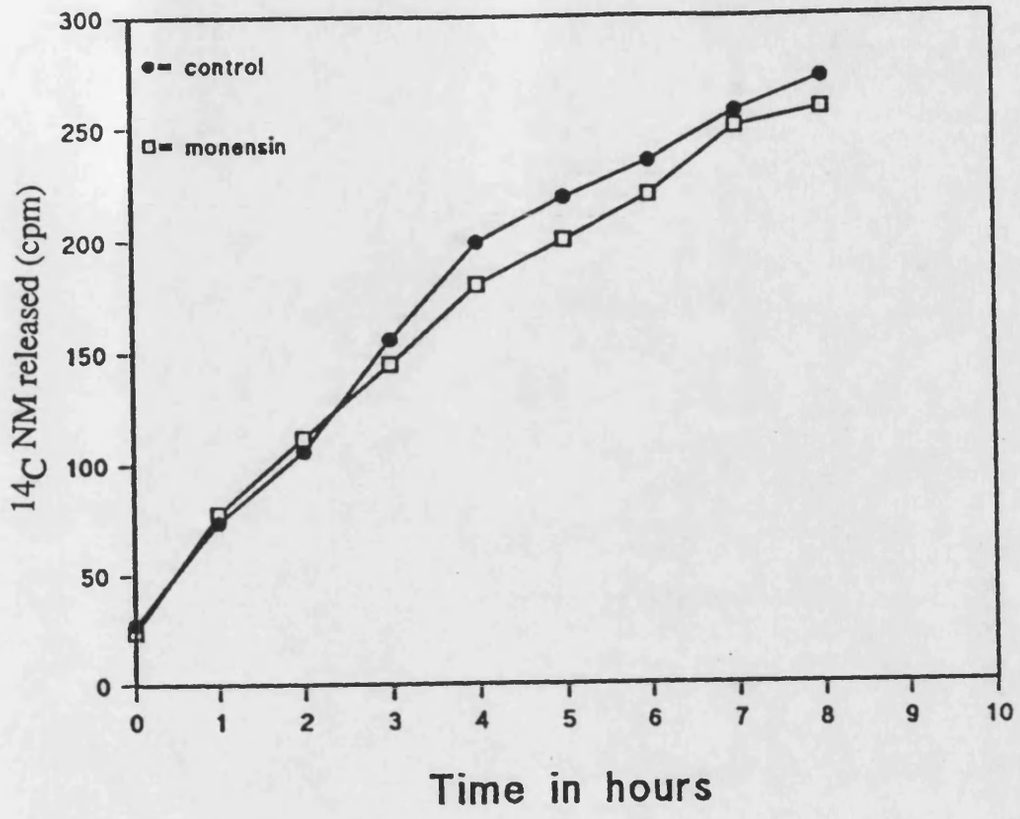


FIGURE 8.7

^{14}C-NICOTINAMIDE (NM) SECRETION FROM L929 CELLS IN THE PRESENCE OR ABSENCE OF MONENSIN (1mg/ml).

L929 cells were metabolically labelled with ^{14}C-Nicotinamide for 48 hours. At this time the labelled medium was carefully replaced with fresh serum free media(with or without monensin) as described in materials and methods. The conditioned medium was collected at different time intervals (time is indicated in the above figure) and scintillation counting was performed. Values on the y-axis are expressed as the percentages of the total uptake of the radioactivity by the cells ($1x10^6$ cells). Further details may be found in the text.

did not take up trypan blue. Moreover, when these cells were labelled with 14C-nicotinamide and the leakage from both control and monensin treated cells was examined, (materials & methods) there was no observable difference in the case of nicotinamide loss from either treated or untreated cells (figure 8.7). That is the monensin does not cause the cells to be leaky.

The enhanced secretion of [^{35}S]-labelled material was an unusual finding, since monensin is considered a useful inhibitor of protein secretion without directly affecting protein synthesis (Mollenhauer et al, 1990). The enhanced secretion was not analyzed further, but would be an interesting subject to follow up at a late date. Nevertheless, the short term treatment with these drugs was not sufficient to impair the strength of L929 cell adhesion

At this stage four questions were asked:

First, does monensin inhibit only fibronectin secretion while the other adhesion proteins were being secreted. Second, is fibronectin secretion only partially inhibited and third, once the final cell adhesion strength is acquired, is it possible to perturb it by simply depriving cells of protein synthesis and secretion. Fourth, do endogeneous proteins play any role if serum is totally eliminated from the culture medium. The following experiments were designed to answer these questions. Thus the second, third and fourth questions were resolved by performing the following experiments.

8.2.3. EFFECT OF PROLONGED TREATMENT OF L929 CELLS WITH EMETINE OR CYCLOHEXIMIDE OR MONENSIN ON THE ADHESION STRENGTH OF THESE CELLS

This experiment was designed for prolonged treatment of L929 cells with the drugs. The object was to eliminate the existing pool of adhesion proteins after protein synthesis inhibition (with emetine/cycloheximide) and completely block the fibronectin secretion (with monensin). At the time of drug treatment cells had been growing for 24 hours (materials and methods).

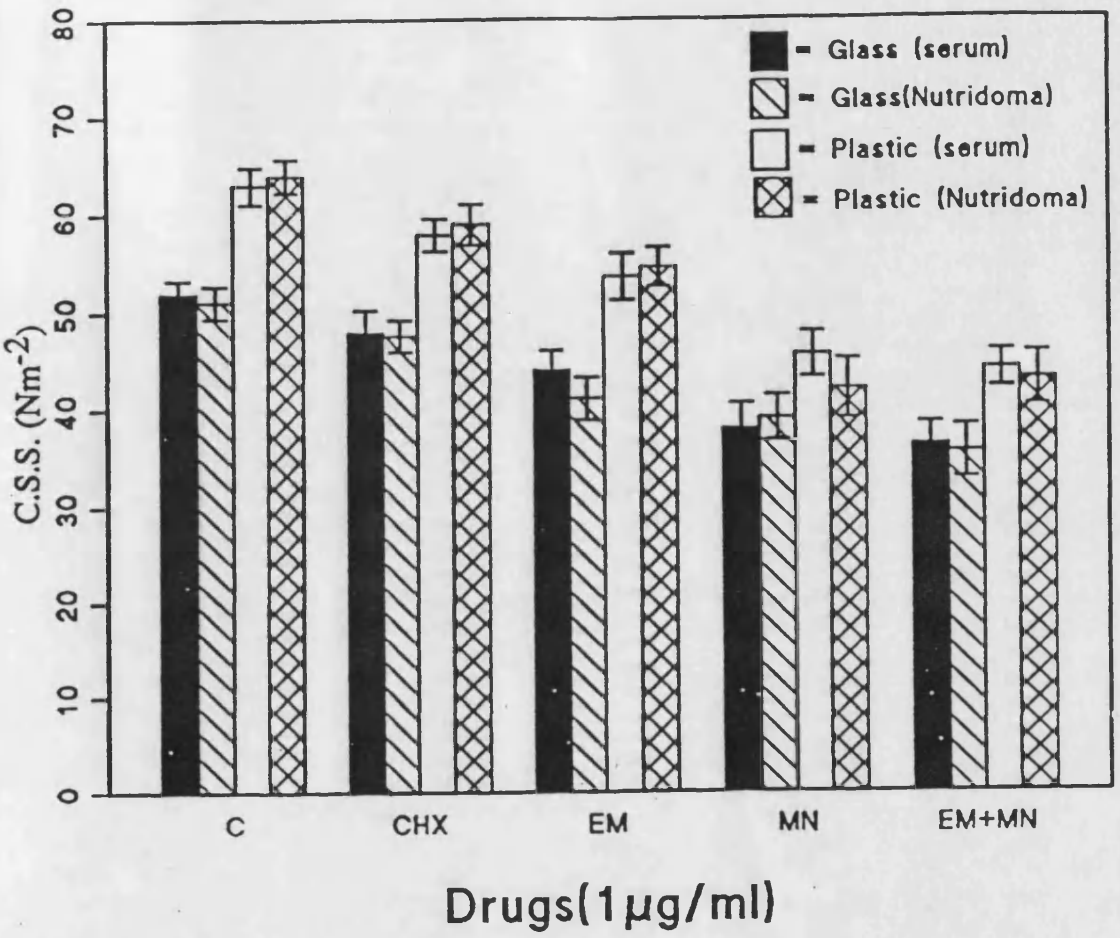


FIGURE 8.8

ADHESION STRENGTH OF L929 CELLS ON GLASS OR PLASTIC SUBSTRATUM IN RESPONSE TO EMETINE (EM) OR CYCLOHEXIMIDE (CHX) OR MONENSIN (MN) OR EMTINE PLUS MONENSIN (EM+M).

L929 cells had been grown for 24 hours and the culture medium was replaced with new culture medium (serum or serum free) containing drugs indicated on the x-axis of this figure. The untreated (C) or treated cells (EM, CHX,MN,EM+MN) were further incubated for 24 hours. At this stage their adhesion strength was measured in terms of the critical shear stress (c.s.s.) of detachment.

Each data point represents the mean of 10 different experiments, in each of which 20 measurements of the c.s.s. were made. The error bars indicate the standard error of that mean.

A t test indicates a significant difference ($p=0.0001$) in the adhesion strength of drug treated or untreated cells. The difference between adhesion strength of L929 cells treated with emetine or cycloheximide is significant ($p=0.0027$). However, the difference between adhesion strength of untreated or treated (with emetine or cycloheximide) is highly significant ($p=0.0001$).

Likewise there is no significant difference ($p=0.21$) in the adhesion strength of L929 cells treated with monensin alone or together with emetine, whereas compare to control the depression of adhesion strength in response to these drugs is highly significant ($p=0.0001$). Further details may be found in the text.

When L929 cells which had been growing for 24 hours were treated with monensin (1μg/ml) alone the c.s.s. was inhibited 27% of the control c.s.s. value of the L929 cells. When the L929 cells were treated with monensin together with emetine (1μg/ml) the critical shear was inhibited 30% of the control shear values of these cells (figure 8.8).As indicated in the figure 8.8 monensin alone significantly affected the adhesion strength of L929 cells over this longer period. That is, it depressed 27% normal adhesion strength of L929 cells. The effect of monensin might be due to the impairment of fibronectin secretion. It is possible that small residual pools of endogenous fibronectin may have been dispatched from the cells before the monensin effectively inhibited secretion of this molecule. This may be the reason that the adhesion strength was not as depressed as expected. This idea gains support from the findings of Curtis (1987) that very small pools of endogenous fibronectin was the minimum requirement for cell adhesion and my results on catalytic quantities of fibronectin (chapter 7).

Although not equivalent to the monensin effect, some reduction in adhesion strength was noted in response to cycloheximide and emetine. As shown in the figure 8.8, emetine and cycloheximide significantly ($p=0.0001$) reduced critical shear values of L929 cells. For example emetin (1μg/ml) depressed 15% and 14% control shear values from plastic and glass substrata respectively. Cycloheximide being less potent than emetine only depressed the adhesion by 8% and 7% of the control shear values from plastic and glass substrata. This may be reflecting the different extent of protein synthesis inhibition in response to these drugs (figure 8.3 & 8.4).

The minor depression in adhesion strength might represent the direct effect of long term protein synthesis inhibition by these drugs. These effects may include the synthesis of adhesion protein(s), receptor(s) and the inability of the cells to complete generation of F-actin stress fibres. (Flinckinger & Culp, 1990). Further this minor reduction does support the idea that once cells acquire their final adhesion strength, protein synthesis in the presence of serum, plays little role in the adhesion of L929 cells.. However, secretion of protein(s) from the cells continues even after protein

synthesis inhibition (Grinnell & Feld, 1979). As stated earlier, this is because of the long cellular half life of fibronectin and fibronectin receptors. For example Ryseck et al (1989) have recently described the isolation of independent clones encoding for interacting components of adhesion system, whose expression is rapidly increased by growth factors even in the presence of cycloheximide. These clones are reported to be encoding for fibronectin, fibronectin receptor and α-actin. The messenger RNA corresponding to each of these moieties showed a long half life and remained at high levels for at least 8 hours. This may be the reason that the effect of these drugs (emetine/cycloheximide) on the adhesion strength was not as much as was expected.

It is clear that the above protein synthesis inhibition studies can not , in themselves, unravel the complexity of cell adhesion strength, once the cells have attached and spread. Thus as revealed in the above findings once the cells acquire their final adhesion strength, although these drugs significantly ($p=0.0001$) depressed it was not as much as one might expect. Therefore, In the next series of experiments the inhibitors were introduced before the cells had developed their adhesion structures.

8.2.4. EFFECT OF EMETINE OR CYCLOHEXIMIDE OR MONENSIN ON THE ADHESION STRENGTH OF L929 CELLS BEFORE THEY DEVELOP THEIR ADHESION STRUCTURE

In this experiment the effect of protein synthesis was checked before the expected development of adhesion structures. For this purpose the inoculation of cells was accompanied by the addition of drugs in the culture medium. The critical shear under these conditions showed interesting results. For example emetine or cycloheximide (1μg/ml) depressed the adhesion strength by 32% and 33% respectively on glass and 33% and 31% respectively on plastic (figure 8.9).

When the L929 cells were treated with monensin (1μg/ml) alone or together with emetine (1μg/ml) at the time of inoculation, the critical shear stress was dramatically decreased. That is monensin alone depressed adhesion strength of L929

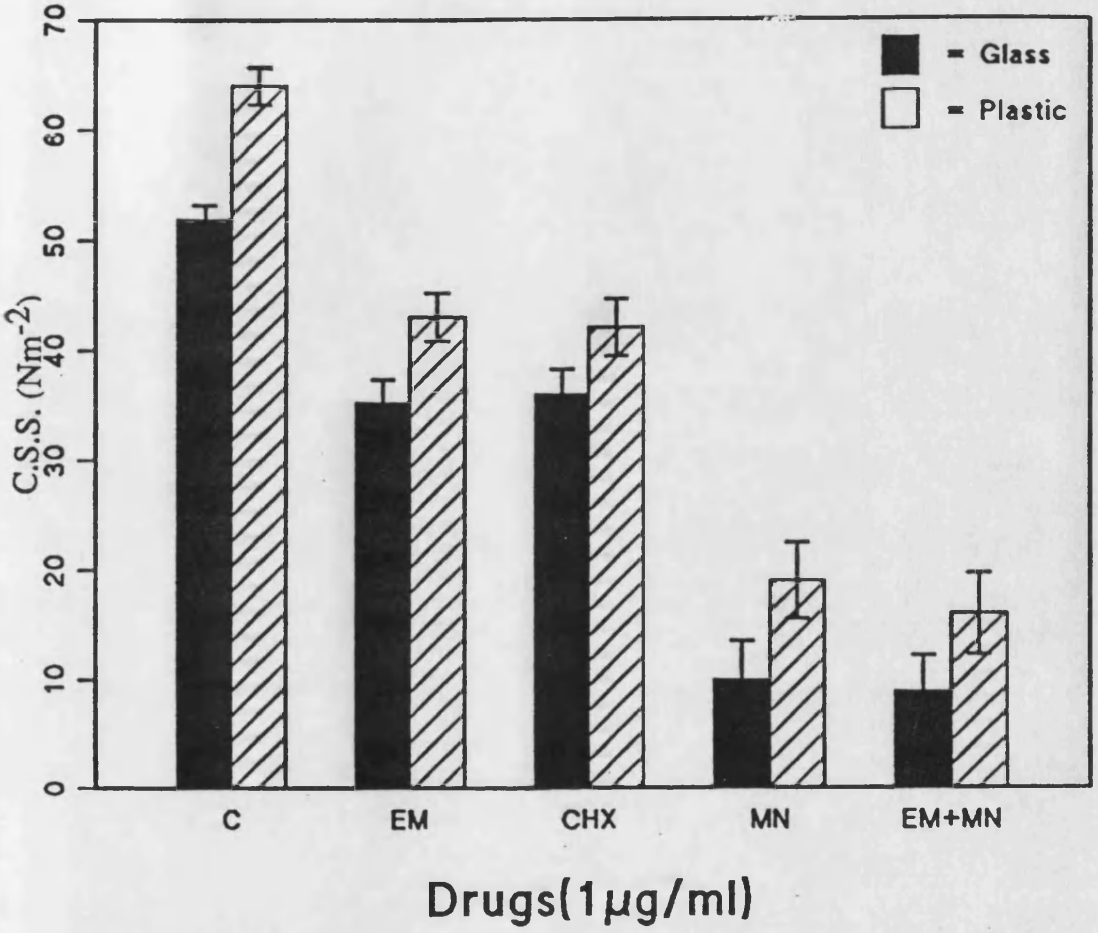


FIGURE 8.9

ADHESION STRENGTH OF L929 CELLS ON GLASS OR PLASTIC SUBSTRATUM IN RESPONSE TO EMETINE (EM) OR CYCLOHEXIMIDE (CHX) OR MONENSIN (MN) OR EMETINE PLUS MONENSIN (EM+MN).

L929 cells were inoculated in the culture medium (containing 10% serum) containing drugs (indicated on the x-axis of this figure) or without drugs. The treated (EM, CHX, MN, EM+MN) or untreated (C) cells were incubated for 24 hours and adhesion strength of these cells was measured in terms of the critical shear stress(c.s.s.) of detachment.

The error bars indicate the standard error of the mean of 10 different experiments, in each of which 20 measurements of c.s.s. were made. The error bars represent the standard error of that mean.

A t test indicates that the difference in adhesion strength of L929 cells treated with emetine and cycloheximide is not significant (p=0.56), likewise the difference between adhesion strength of L929 cells treated with monensin alone or together with emetine is not significant (p=0.06). However, compared to the untreated cells all drugs depressed adhesion strength highly significantly (p=0.0001).

cells by 75% and 70% respectively on glass and plastic substrata, whereas when it was used with emetine it decreased adhesion strength of these cells by 78% and 72% respectively on glass and plastic substrata (figure 8.9) It shows that the main effect was due to monensin. This overall and quite dramatic effect on the critical shear stress of detachment was significant to p= 0.0001 (figure 8.9). These experiments were repeated with pre treatment of the drug regimes (stated above) for 48 hours and no further decay in adhesion strength occurred compared to the 24 hours period. At each of these periods the viability of the cells was checked with trypan blue exclusion (materials & methods) and always showed better than 98.8% viability. These results indicate that L929 cells are largely dependent on endogenous fibronectin to achieve their final adhesion strength. This idea may be favoured by the fact that monensin is a specific inhibitor of fibronectin secretion and this may have caused a substantial reduction (70%-75% of the control value) of the adhesion strength of the L929 cells.(figure 8.9)

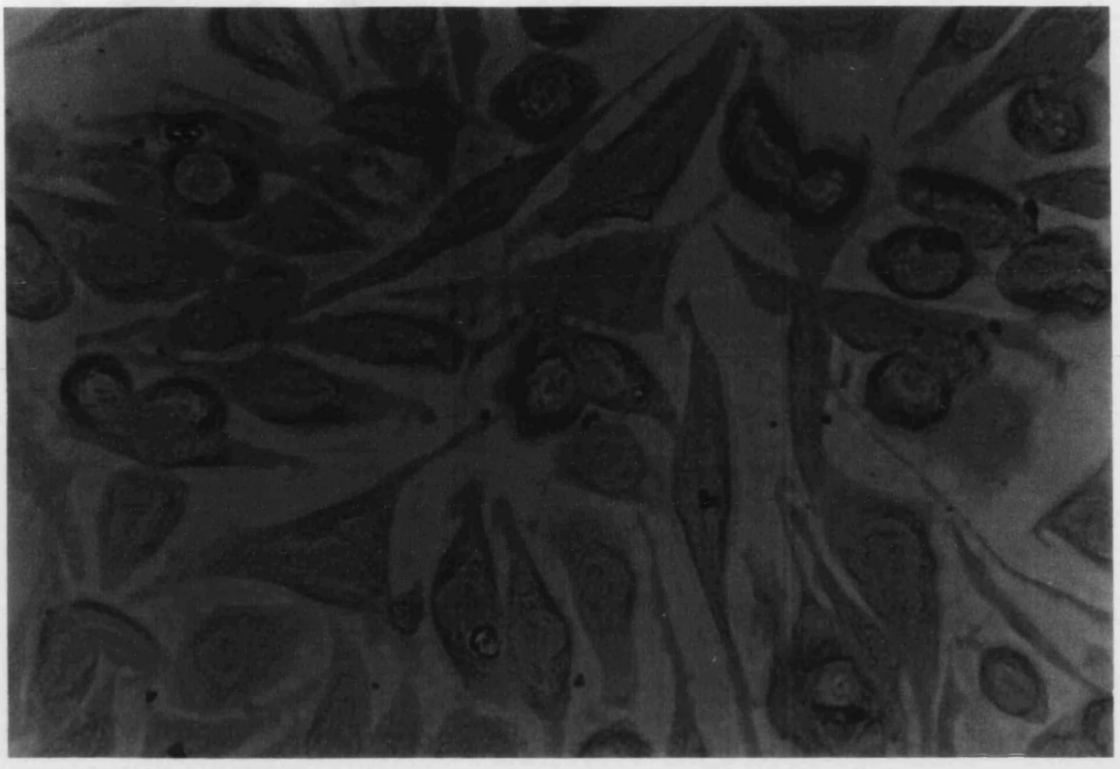
Although the decrease in adhesion strength of L929 cells in response to emetine or cycloheximide is significant (p=0.0001), these inhibitors unlike monensin, failed to exert a large effect on the adhesion strength of these cells. Again the reason may be that a small pool of fibronectin is retained by the cells which they secrete later and utilize for their adhesion (Grinnell & Feld, 1979, Curtis, 1987). Treated or untreated cells are shown in the pictures 8.1, 8.2 and 8.3.

As already mentioned externally introduced proteins in the cell culture system might send signals to the interior of cells for achieving their final adhesion strength. The results of these experiments showed that this signal may be stimulating protein synthesis and secretion. The final adhesion strength can only be achieved after the transmission of signals to the interior of cells by both the exogenous and endogenous proteins. In fact a 70-75% reduction in adhesion strength in response to monensin is a strong hint for the importance of endogenous protein(s) involvement in the adhesion strength of L929 cells. It may be that monensin blocked the secretion of endogenous fibronectin and prevented it from becoming part of the extracellular matrix. There are

some published reports that at 10% serum it is vitronectin which mediates cell adhesion (Knox, 1984; Underwood & Bennet, 1989). Since in the present study 10% serum containing culture medium was used, the presented results imply that when vitronectin is available from the serum the adhesion strength of L929 cells requires endogenous fibronectin. The secretion of the latter was inhibited by monensin which probably caused a severe depression of the adhesion strength (70%-75%) in this experiment (figure 8.9).

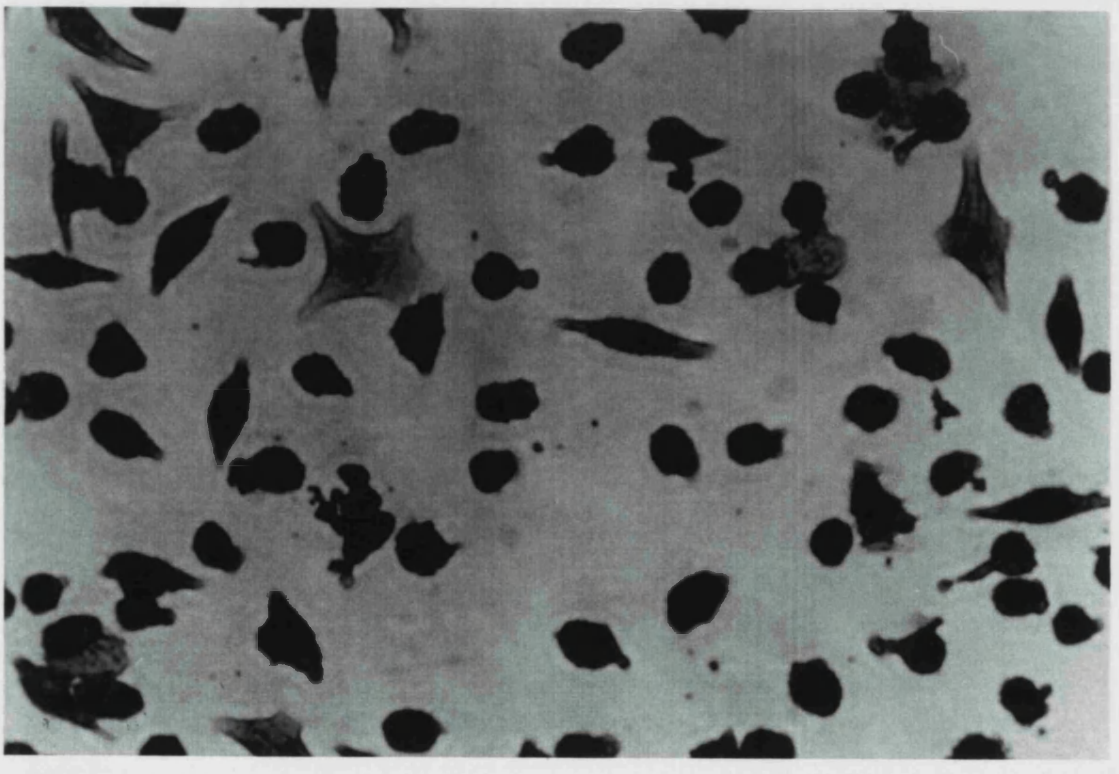
The results presented here indicate clearly that even in the presence of serum, cells do not enter into the proposed second phase of the adhesion strengthening when monensin is present. That is, no significant difference between the adhesion strength of cells growing in the medium containing serum (with monensin) and of those which were growing in serum free media (without monensin) was observed. One interpretation of this is that externally added proteins may transmit signals to the cells to trigger the secretion of fibronectin. The latter finally becomes part of the extracellular matrix which in turn is responsible for the adhesion strength of L929 cells or in other words L929 cells depend on the endogenous fibronectin for their entrance into the proposed second phase of the cell adhesion.

Up to this point the adhesion proteins have all been assumed to have positive adhesion, that is, they increase cell-substrate binding. This may not be the case. Some proteins have been proposed to have anti-adhesive properties (Faissner & Kruse, 1990). It is possible that these might show enhanced secretion in the presence of monensin, thus causing the low adhesion strength. Such proteins (tenascin) are very poorly understood and almost uncharacterized. (Faissner & Kruse, 1990). However the emetine plus monensin experiment probably rules this possibility out. As shown earlier, emetine is an extremely powerful inhibitor of protein synthesis and so if monensin was enhancing the secretion of antiadhesive proteins it could only be from a pool of pre-existing molecules. Unless, again an unlikely proposition, the antiadhesive protein synthesis is not sensitive to emetine.



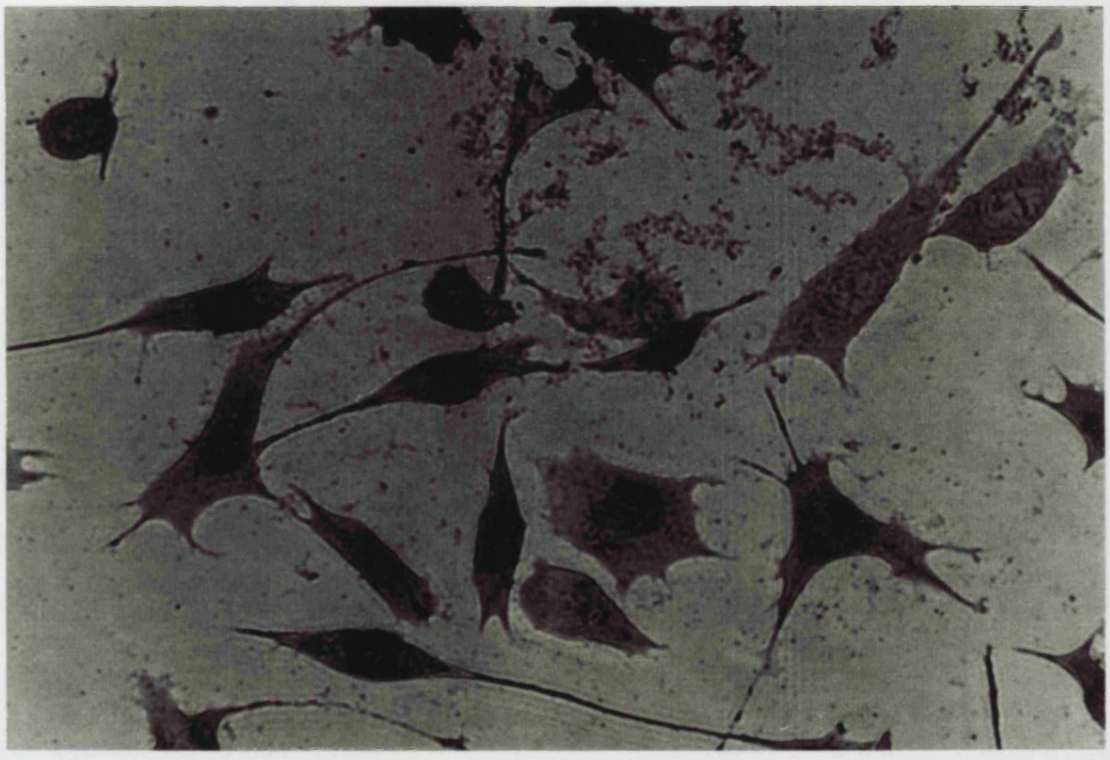
PICTURE 8.1

Normal untreated L929 cells



PICTURE 8.2

Emetine treated L929 cells



PICTURE 8.3

Monensin treated L929 cells

It is possible that in the presence of monensin L929 cells might have ceased to develop the stress fibres and adhesion plaques which were speculated to participate in the adhesion strengthening phenomena. (chapter 3).. Of course in these circumstances the cells would not be able to develop their final adhesion strength It remains to be determined how much fibronectin synthesis was inhibited by the monensin. Thus an acknowledged weakness in these proposals is whether or not the monensin was an effective inhibitor of fibronectin secretion. Published data confirms that it is effective but ideally, confirmation should have been sought that the inhibition was absolute. Unfortunately a fibronectin monoclonal (specific) antibody was not available for this study. Considering reports in the literature one can be reasonably certain that the bulk of the endogenous fibronectin did not participate in the adhesion strength of L929 cells in the presence of monensin.

The release of endogenous fibronectin may not be obligatory for every cell type. For example, a murine cell strain NALIA (C4 clone) was shown to be dependent on a vitronectin coated substratum for its attachment. Moreover, it did not need endogenous protein synthesis for adhesion (Steele et al, 1991). However, this latter study was qualitative with respect to attachment and these researchers did not check whether protein synthesis is required or not for the development of final adhesion strength in murine cells. However, at present it can not be generalised that fibronectin synthesis and secretion is necessary to achieve final adhesion strength of each cell type. So far, the possible effect of monensin on the secretion of fibronectin has been discussed in great detail. However it must be remembered that fibronectin as a ligand is only half of the interaction. The other half is the fibronectin receptors. Many reports suggest that the total cell surface receptors are in large excess over the number that are actually used for adhesion (Brown & Juliano, 1987). Thus it seems extremely unlikely that the fibronectin receptors are effected by monensin.

It is revealed in the figure (8.3 & 8.4) that the response to these drugs needs 8 hours or more for substantial protein synthesis inhibition. Moreover, these studies were carried out in the presence of serum in growth medium which is a source of

adhesion protein(s). It was thought that during these 8 hours secreted protein(s) together with adsorbed serum protein(s) might have established certain links with the cell machinery via integrin receptors, therefore, the total adhesion strength was not inhibited. To abolish this possibility the next series of experiments was designed to rule out the role of serum. That is, until now, the discussion has been about experiments which were performed in the presence of serum. From now on the experiments are carried out in the absence of serum. These are discussed below.

8.2.5. ADHESION STRENGTH OF L929 CELLS IN RESPONSE TO EMETINE, CYCLOHEXIMIDE OR MONENSININ IN SERUM FREE MEDIA

To assess the strength of cell adhesion when solely determined by endogenous proteins, L929 cells were sub cultured in 1% Nutridoma (materials & methods) with or without drugs and incubated for 24 hours. After this time all the cells lost their adhesiveness and simply detached from the substratum, indicating that cell adhesion is an active phenomena which needs continuous on going protein synthesis in the absence of serum.

As mentioned above and indicated in table 8.1, treatment of cells with emetine or cycloheximide or monensin completely abolished the adhesiveness of L929 cells to both glass and plastic. The cells initially attached in the presence of drugs in serum free medium and retained their anchorage for 8 hours and thereon gradually lost their adhesiveness. The viability of cells was checked with trypan blue exclusion method and it was found that they did not uptake trypan blue, therefore, their viability was considered intact (98%). The findings of this experiment indicate that cell adhesion is not a passive process as was suggested by others (Neumeier & Reutter, 1985) because for continued cell adhesion, on going protein synthesis was required.

The question arises that if after protein synthesis inhibition, a small intracellular pool of adhesion proteins exist (as suggested earlier in this chapter), why did it not take part in the adhesion of L929 cells. The possible answer is that cells

Drug treatment	Glass	Plastic
	C.S.S(N/m2)	C.S.S(N/m2)
Control	13.0 ± 3.5	9.1 ±4
Emetine	0	0
Cycloheximide	0	0
Monensin	0	0
Monensin + Emetine	0	0

TABLE 8.1

L929 cells were grown in serum free medium with or without drugs (mentioned in the table. Results show that in drug containing cultures cells lost their adhesiveness but in the absence of drugs cells showed measurable adhesion on both glass and plastic substratum.

might have secreted protein(s) which caused adhesion but in the absence of added serum proteins there may be very weak or no stimulation of secretion of adhesion proteins. Since there was no serum and new protein synthesis was inhibited, the adhesion structures were more prone to proteolysis. For example Werb et al (1989) revealed that preventing the actual ligand binding with receptors could induce the expression of proteolytic enzymes. Similar expression of proteolytic enzymes may be involved to disrupt the adhesion structures of drug treated cells in serum free media.

Where drug treated cells in serum free media lost their adhesiveness, the untreated cells in serum free media showed some interaction strength to the plastic ($9.1 \pm 4 Nm^{-2}$) and glass (13.0 ± 3.5 Nm^{-2} table 8.1). Again comparatively large standard deviation specific to serum free media indicated the heterogenity among the sub populations of L929 cells. The efficiency of the Microflow chamber that made a valuable distinction between such sub populations Moreover it is the Microflow chamber with the help of which cell adhesion has proved to be an active process rather than passive one because protein synthesis is required to strengthen the cell adhesion. Thus it will be inappropriate to equate this phenomena to the wetting of a surface by a fluid droplet (Forrester & Lackie, 1984).

Finally the findings of this chapter can be summarized as follow

(a) The L929 cells growing for 24 hours are not responsive to 3 or 6 hour drug treatment

(b) A minor but statistically significant effect in terms of the c.s.s. of detachment was noted when L929 cells have been grown for 24 hours. Here monensin considerably depressed the c.s.s. of detachment of L929 cells by 30% of the control value.

(c) A considerable reduction (40% of the control value) in adhesion strength occurred when emetine or cycloheximide were added at the time of inoculation of the cells. In

contrast to these drugs, monensin alone or together with emetine substantially depressed the adhesion strength of L929 cells by 70%-78% of the control value.

(d) When the drugs were added at the time of seeding of cells in serum free medium, it was of interest to note that cells automatically started to detach from the surface and after 24 hours their adhesiveness was completely abolished.

8.3 CONCLUSION

In conclusion, serum proteins cause an initial attachment and then perhaps send signals to the interior of cells to enhance protein synthesis and secretion. These endogenous protein(s) in turn attach to the receptors and this attachment facilitates the orgnisation of cytoskeleton and adhesion plaque formation. This conclusion is drawn because when fibronectin secretion was inhibited with monensin in presence of serum the adhesion strength of L929 cells was substantially depressed(70% to 78%). In serum free media the inhibition of protein synthesis abolishes the adhesiveness of L929 cells completely indicating that their adhesion is an active process.

Further work is needed to transform these conclusions from educated speculations to a solid factual basis.

CHAPTER 9

GENERAL DISCUSSION & CONCLUSION

The research presented here had as its objectives "the development of a simple and reproducible cell adhesion measurement device and its applications to understand the underlying mechanism of cell adhesion and cell adhesion strengthening phenomena. This was necessitated due to the following reasons.

In previous experimental protocols cells are permitted to interact with the culture vessel and adhesion is measured by quantifying the number of cells that bind to the surface in a given time period. Clearly, any attempt to maximize the use of cell adhesion in various fields (from biotechnology to clinical research) demands an understanding of the principles and mechanisms involved in the attachment and stabilization of cell adhesion. While the previous techniques, being qualitative methods, can assess the former with a varying degree of success, the latter remained totally unresolved. Some methodologies for qualitative assessment of cell adhesion have been developed which were either unable to measure the stabilized adhesion (final adhesion strength) or they have run into several other obstacles (indicated in chapter 1 & 3). That is all these techniques suffered from the need for complex equipment, whereas the technique should not only be simple but also should yield reproducible and usable data for assessment of quantitative cell adhesion. These requirements are largely met in the present study with the development of a cell adhesion measuring device or "Microflow chamber".

The Microflow chamber not only measures the final strengthened adhesion but also is a simple and reproducible technique. At present two designs of Microflow chamber exist. The first design was limited in its capacity to measure the critical shear stress (c.s.s.) of detachment of cells from glass microscope slides. The second of my designs could measure the c.s.s. of detachment from glass and plastic petri dishes. Both designs are simple and easy to handle. One of the main advantages of this system lies in the fact that the physical conditions of the detachment assay are completely defined. Contrary to the previous techniques, the convergent channel of

the Microflow chamber increases the velocity which gives an increasingly smooth or laminar flow (chapter3). Thus the formula developed to calculate the c.s.s. of detachment remains valid even at a very high flow rate. In fact under precise hydrodynamic control a range of well defined shear forces over the cells could be set up. This feature of the Microflow chamber is predominantly different from previous techniques.

Having developed this device, its accuracy and reproducibility was checked by measuring adhesion strength of different cell lines on the same or on a series of different surfaces, ranging from glass to fibronectin modified plastic. The results obtained were rather a mirror image of the theoretical predictions about well defined forces in the convergent channel of the Microflow chamber. This is obvious in the context of the very small obtained standard deviation in each experiment carried out in the present study. The sophistications of this device can be seen by its sensitivity for recognising two systems which are only slightly different with respect of their environment. Using existing techniques there may appear to be no difference between two systems, but the Microflow chamber can detect it by providing results with a statistical significance of p=0.0001 (chapter 5).

Thus the device not only can be used to compare cell adhesion to a series of surfaces in a quantitative way, it is a useful tool for understanding the cell adhesion strengthening phenomena. For example, the adhesion strength (in term of c.s.s.) of different cell lines on different surfaces show unambiguously that cells are held to a surface with a certain fixed strength. Once this is exceeded by an applied force, the cells will be removed from the surface. This force is termed the critical shear stress of detachment. The results obtained in the present study with regard to different cells and surfaces also indicates that a critical shear stress of detachment is a general phenomena occurring with all cells and all surfaces. This generalisation gets support from the findings that every cell line showed its specific and constant critical shear value with respect to the surface on which it is growing.

The most encouraging finding with the Microflow chamber was its high reproducibility as was shown by measuring the c.s.s. of detachment of many cell lines over a number of passages. However, some cell lines showed ageing with respect of c.s.s. of detachment. Even these cells , whenever taken from liquid nitrogen always show a constant and specific critical shear value indicating the high reproducibility and sophistication of the Microflow chamber. It is of great interest that animal cells actually possess such a specific adhesion value. It also strongly implies that different cell lines have different adhesion mechanisms, since they show such differing adhesion constants. Thus the major finding in this work is that, for particular established cells,the adhesion strength is a constant especially if that value is measured soon after establishment of the culture from liquid nitrogen. It is to be hoped that primary cell lines will show a similar constant for each specific line. Indeed it is to be expected that the adhesion strength in such cells is to be closely related to the organs or the tissues from which they arose. That is, there may be a relationship between adhesion and differentiation.

It is not only that a device should be able to measure cell adhesion quantitatively but it must also be able to evaluate the physical, biological and environmental factors involved in cell adhesion strengthening phenomena. This system should be amenable to investigation of the parameters causing the effects on cell adhesion. It is very pleasing that this Microflow chamber can evaluate the role of different parameters contributing in a complex cell adhesion phenomenon.

The complexity of the cell adhesion is due to numerous parameters acting directly or indirectly in gaining the final adhesion strength of a cell. The factors examined with the help of the Microflow chamber include the cells themselves, different substrates, serum, purified adhesion proteins and finally the endogenous adhesion proteins. Before examining these parameters there was a question in mind that asked "what is the mechanism involved in the specific adhesion strength of a particular cell line in a similar environment".

The spreading of the cells was considered as a suspected factor for specific adhesion strength. That is the bigger the cell the more force may be required to detach it from the surface. In fact it was found that adhesion strength is not solely determined by the spread area of the cell. This finding was contrary to some theoretical studies which emphasized that adhesion strength is dependent on the surface area of the cell. Although in the present work the surface area of cells was not measured quantitatively the qualitative observations under an inverted microscope suggest that big cells can have a lower adhesion strength than a smaller cells (chapter 4 & 5). Moreover, changing one parameter (e.g. serum) and keeping others constant may not have any impact on cell spreading but it may have a profound effect on the adhesion strength of cells (chapter 5).

Indeed this finding led me to propose the two phases of cell adhesion which until now has been considered to simply be a single step mechanism involving an attachment which leads continuously to spreading. The present study proposes that attachment and spreading are different aspects of, or different steps within, cell adhesion but still this is not a complete description of this intricate phenomena. In fact attachment plus or minus spreading is the first phase of cell adhesion which activates the events leading to the specific, final, strengthened adhesion (second phase). The present work was not aimed at the elucidation of how these events are activated, however, evaluation of different parameters with the help of my Microflow chamber strongly pleads the case for protein-receptor bond involvement in this phenomena.

These bonds may be participating directly or they may be activating some unknown molecule(s) in the interior of the cell (this is not the direct subject of the present study). If these bonds are involved, whatever the mechanism, it is conceivable that by preventing these bonds from forming causes the adhesion strength of cells to be depressed. Indeed this was proved to some extent when the synthetic peptide RGDS, a recognition sequence, mimicked the adhesion strength of cells at least partially (chapter 6). It was again very pleasing that the Microflow chamber can assess and quantify the reactions which occur at the receptor level. That is, upon

addition of RGDS to the culture medium a measurable and significant (p=0.0001) depression of adhesion strength was observed (chapter 6). This effect was attributed to the protein-receptor bond involvement because it is now well known that cells interact with the RGDS cell attachment sites of the various adhesion proteins through cell surface receptors. Since adhesion strength was not fully depressed, this finding suggests a versatile mechanism which cells can use for their specific adhesion strength. This notion is made because the incomplete reduction of adhesion strength was attributed to RGDS independent sequences within the fibronectin molecule or in other proteins. Nevertheless, the refinements of the Microflow chamber were obvious, since it was able to recognise the dose dependent impact of RGDS on cell adhesion strength.

These findings also show that RGDS is not indeed all that is required for the receptor binding. Further studies are needed to show the exact quantitative participation of RGDS dependent and independent sequences in the cell adhesion strengthening phenomenon. However, determining the exact quantitative share of each sequence may be interesting but is outside the scope of this thesis, since the aim in this experiment was only to check whether protein–receptor bonds are involved in adhesion strengthening or not. Of course, the major aim was to check that the Microflow chamber could analyse and show a distinction between two systems i.e. normal culture medium and RGDS containing culture medium. The next thought was to see if these bonds are involved in the adhesion strengthening phenomenon. Thus it was worthwhile to check the effects of a specific protein on the adhesion strengthening phenomena.

Two approaches were made to examine the effect of fibronectin, a prototype adhesion protein, on cell adhesion strength. That is the role of exogenous and endogenous fibronectin in adhesion strengthening phenomena was evaluated.

In the first attempt surfaces were modified with fibronectin and cells were grown in the serum free medium. Interestingly the adhesion strength was again found to be substrate dependent, that is cells adhere with varying strength to different

surfaces. For example L929 cells showed comparatively low adhesion strength on glass than on plastic and the highest adhesion strength on fibronectin modified surfaces. These findings support an earlier proposal made in this discussion that critical shear stress may be a general phenomenon occurring with all cells and surfaces.

As was mentioned earlier (chapter 7), a very exciting and another major finding in this work was that fibronectin coating on plastic substrates dramatically enhanced the adhesion strength of L929 cells. To date fibronectin has been proposed as a major adhesion molecule. In the previous works, the assumption has been made that it is a mediator between the surface of the cell and the surface of the substratum. Another way to evaluate the role of fibronectin is that, within certain limits the more fibronectin there is adsorbed to the surface the stronger the adhesion strength will be. This is true especially, if one believes that adhesion strength is dependent on the number of adhesion bonds (theoretical studies cited in chapter 1).

With the successful development of the Microflow chamber this is now a testable hypothesis. Does cell adhesiveness follow a straightforward dose dependency with respect to fibronectin?. Thus it can now be checked whether or not the adhesion strength is directly related to the number of fibronectin molecules on the surface. As can seen from the chapter 7, fibronectin in small amounts has a quite unexpectedly dramatic effect on the adhesion strength of L929 cells. It certainly does not appear that fibronectin is a passive mediator. Since only 2000 molecules per cell (pre-adsorbed to the surface) increase adhesion strength by 9 fold. Of course the number of molecules per cell is a calculated value and open to some criticism. However, as mentioned in the results (chapter 7) there were a maximum of 2000 molecules of fibronectin/10μm^2 of substrates, causing an adhesion strength (82.5 Nm^{-2}) 9 fold more than the normal value.

The strength was not increased if greater than 2000 molecules/cell were used. Though entirely unexpected, this finding caused the question of how such a very small number of molecules could have such a profound effect on the adhesion

strength. Although this work was not designed to answer this question, the findings do open the real possibility that the fibronectin is a signalling system or at least a part of a signal. How these signals transmitted or controlled is unknown but some discussion of this can be found in chapters 4 & 7.

The immediate intriguing question was what is the end product of this suspected signalling mechanism. Among other suspected molecules were endogenous proteins. This choice was indeed fitting in the framework of this thesis. To this end again with the courtesy of the Microflow chamber, the effect of the synthesis and release of the endogenous adhesion proteins on adhesion strength was examined. (chapter 8).

The previous studies in the literature though qualitative were focused on the effect of protein synthesis on cell adhesion. To date no attempt has been made to check the effect of secretion on cell adhesion. The results presented in chapter 8 suggest that protein synthesis inhibition at the time of the experiment may not be sufficient since already synthesized proteins may be secreted and utilized for cell adhesion. Therefore, the inhibition of secretion was desirable. The overall inhibition of the secretory mechanism may not be helpful since one condition of the adhesion assay is to keep cells as healthy as possible. Therefore, a specific agent which could block the secretion of some adhesion proteins was required. Since fibroblasts are reported to secrete fibronectin but not vitronectin (Preissner, 1991), it was logical to inhibit the secretion of fibronectin. It followed that monensin was such a chemical which was reported to inhibit secretion of fibronectin.

Thus with the disruption of fibronectin secretion, 75% of the normal adhesion strength of L929 cells was lost (or L929 cells showed only 25% of the normal adhesion strength). These results indicate that to attain the final adhesion strength these cells are dependent on fibronectin secretion. It also therefore, implied that protein-receptor bonds may still be relevant for the induction of the adhesion because the recruitment of the fibronectin receptor may be driven by the release of fibronectin whose secretion was blocked by exposing cells to the monensin. Of course this

conclusion is based on the numerous reports published that monensin specifically impedes secretion of fibronectin. What is the fate is of the other adhesion molecules in monensin treated cells has yet to be determined.

These findings were also supportive for the idea of the proposed two phases of cell adhesion. That is although monensin treatment does not exert any effect on spreading, it dramatically depressed the adhesion strength. This leads to speculations that cells can interact with any adhesion protein present in the serum, but this interaction might only establish low adhesion strength bonds (first phase) that cannot account for final adhesion strength. The release of fibronectin may recruit fibronectin receptors which are responsible for final adhesion strength (second phase). However, to date, the participation of other adhesion molecules in the complex phenomenon of adhesion strength cannot be excluded.

Although the work presented here is preliminary, it suggests that if a second messenger system is operative it must be linked with protein synthesis and secretion. Indeed these results suggest the beginning of an enormous breakthrough in establishing adhesion by this adhesion protein activated second messenger system.
To elucidate this system is be the task of the future, since the Microflow chamber is available. The data obtained in this thesis will help to find out about and to discover the second messenger molecule in cell adhesion.
In conclusion, the successful development of the Microflow chamber opened a enormous vista of possible work in cell adhesion which will help us to understand the underlying mechanism of cell adhesion.

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